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<b>(54) Title:</b> VARIANTS OF BILE SALT-STIMULATED LIPASE, DNA MOLECULES ENCODING THEM, AND TRANSGENIC NON-HUMAN MAMMALS  <b>(57) Abstract</b>  <p>The present invention relates to novel polypeptides which are variants of Bile Salt-Stimulated Lipase (BSSL; EC 3.1.1.1). It also relates to DNA molecules encoding the said polypeptides, and to subproducts comprising the said DNA molecules. The invention further relates to processes for producing the said BSSL variants and for producing transgenic non-human mammals capable of expressing the BSSL variants. Furthermore the invention relates to such transgenic animals as well as to infant formulas comprising milk from such transgenic animals. The invention also relates to pharmaceutical compositions comprising the said polypeptides; and the use of the said polypeptides and DNA molecules for the manufacture of medicaments.</p>		

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VARIANTS OF BILE SALT-STIMULATED LIPASE, DNA MOLECULES  
ENCODING THEM, AND TRANSGENIC NON-HUMAN MAMMALS

## TECHNICAL FIELD

5 The present invention relates to novel polypeptides which are variants of Bile Salt-Stimulated Lipase (BSSL; EC 3.1.1.1). It also relates to DNA molecules encoding the said polypeptides, and to subproducts comprising the said DNA molecules. The invention further relates to processes for producing the said BSSL variants and for producing transgenic non-human  
10 mammals capable of expressing the BSSL variants. Furthermore the invention relates to such transgenic animals as well as to infant formulas comprising milk from such transgenic animals. The invention also relates to pharmaceutical compositions comprising the said polypeptides; and the use of the said polypeptides and DNA molecules for the manufacture of  
15 medicaments.

## BACKGROUND ART

20 *Hydrolysis of dietary lipids*

Dietary lipids are an important source of energy. The energy-rich triacylglycerols constitute more than 95% of these lipids. Some of the lipids, e.g. certain fatty acids and the fat-soluble vitamins, are essential  
25 dietary constituents. Before gastro-intestinal absorption the triacylglycerols as well as the minor components, i.e. esterified fat-soluble vitamins and cholesterol, and diacylphosphatidylglycerols, require hydrolysis of the ester bonds to give rise to less hydrophobic, absorbable products. These reactions are catalyzed by a specific group of enzymes called lipases.

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In the human, the essential lipases involved are considered to be Gastric Lipase, Pancreatic Colipase-Dependent Lipase (hydrolysis of tri- and

diacylglycerols), Pancreatic Phospholipase A2 (hydrolysis of diacylphosphatidylglycerols) and Carboxylic Ester Hydrolase (CEH) (hydrolysis of cholesteryl- and fat soluble vitamin esters, but also tri-, di-, and monoacylglycerols). In the breast-fed newborn, Bile Salt-Stimulated Lipase (BSSL) plays an essential part in the hydrolysis of several of the above mentioned lipids. Together with bile salts the products of lipid digestion form mixed micelles or unilamellar vesicles (Hernell et al., 1990) from which absorption occurs.

#### 10 *Bile Salt-Stimulated Lipase*

Bile Salt-Stimulated Lipase (BSSL) is a constituent of milk in a limited number of species, e.g. humans, gorillas, cats and dogs (Hernell et al., 1989, Hamosh et al., 1986). When mixed with bile in upper small intestinal contents, BSSL is specifically activated by primary bile salts (Hernell, 1975). BSSL, which accounts for approximately 1% of total milk protein (Bläckberg & Hernell, 1981), is not degraded during passage with the milk through the stomach, and in duodenal contents it is protected by bile salts from inactivation by pancreatic proteases such as trypsin and chymotrypsin.

Heat treatment of human milk (pasteurization at 62.5°C, 30 min), which inactivates BSSL completely (Björkstén et al., 1980), reduces the coefficient of fat absorption by approximately 1/3 in preterm infants (Williamson et al., 1978, Atkinson et al., 1981). Hence, the superior utilization of fresh human milk triacylglycerol compared to that of infant formulas of similar fat composition is due to BSSL (Hernell et al., 1991, Chapell et al., 1986).

BSSL is a non-specific lipase (EC 3.1.1.1) in as much as it hydrolyses not only triacylglycerol but also di- and monoacylglycerol, cholesteryl esters and fat-soluble vitamin esters (Bläckberg & Hernell, 1983). Thus, after activation, BSSL has the potential to hydrolyze most human milk lipids by

itself, albeit the most efficient utilization of human milk triacylglycerol requires the synergistic action of gastric lipase (EC 3.1.1.3), colipase-dependent pancreatic lipase (EC 3.1.1.3), and BSSL (Bernbäck et al., 1990).

5 Recent studies suggest that the milk enzyme is of particular importance for the utilization of long-chain polyunsaturated fatty acids by the newborn infant (Hernell et al. 1993). These fatty acids are important precursors of eicosanoids and for the neuro-development. Newborn infants, particularly if born before term, have a limited capacity for synthesis of these fatty  
10 acids from their precursors. Hence, they are considered essential for an as yet not defined period of time after birth.

In recent studies from several laboratories the cDNA structures from both the milk lipase and the pancreas Carboxylic Ester Hydrolase (CEH) (E.C.  
15 3.1.1.1) have been characterized (Baba et al., 1991; Hui et al., 1991; Nilsson et al., 1990; Reue et al., 1991) and the conclusion is that the milk enzyme and the pancreas enzyme are products of the same gene. The cDNA sequence and deduced amino acid sequence of the BSSL/CEH gene (SEQ ID NO:1) are disclosed also in WO 91/15234 (Oklahoma Medical Research  
20 Foundation) and in WO 91/18923 (Aktiebolaget Astra).

BSSL is a single-chain glycoprotein. The deduced protein (SEQ ID NO:3) contains 722 amino acid residues and is highly glycosylated (Abouakil et al., 1989). The N-terminal half of the protein shows a striking homology to  
25 acetyl cholinesterase and some other esterases (Nilsson et al., 1990).

A tentative active site serine residue is located at serine-194; the sequence around this serine accords with the consensus active-site sequence of serine hydrolases. The single tentative N-glycosylation site is positioned only  
30 seven residues N-terminal of the active site serine (Nilsson et al., 1990).

The BSSL sequence contains in its C-terminal part 16 proline-rich repeats of 11 amino acid residues each. A variation in number of repeats seems to be a major explanation for differences in molecular size and amino acid composition between corresponding enzymes from different species (Han et al., 1987, Fontaine et al., 1991, Kyger et al., 1989). These repeats carry most of the 15-20% carbohydrate of the protein (Baba et al., 1991, Abouakil et al., 1989).

The unique structural difference between BSSL and typical esterases resides in the C-terminal part of the polypeptide chain, i.e. the 16 proline-rich repeats of 11 amino acid residues. The corresponding pancreatic enzymes from cow and rat have only 3 and 4 repeats, respectively (Han et al., 1987, Kyger et al., 1989). A likely hypothesis has therefore been that the C-terminal part, or at least part of it, is indispensable for lipase activity, i.e. activity against emulsified long-chain triacylglycerol.

#### *Lipid malabsorption*

Common causes of lipid malabsorption, and hence malnutrition, are reduced intraluminal levels of Pancreatic Colipase-Dependent Lipase and/or bile salts. Typical examples of such lipase deficiency are patients suffering from cystic fibrosis, a common genetic disorder resulting in a life-long deficiency in 80% of the patients, and chronic pancreatitis, often due to chronic alcoholism.

The present treatment of patients suffering from a deficiency of pancreatic lipase is the oral administration of very large doses of a crude preparation of porcine pancreatic enzymes. However, Colipase-Dependent Pancreatic Lipase is inactivated by the low pH prevalent in the stomach. This effect cannot be completely overcome by the use of large doses of enzyme. Thus the large doses administered are inadequate for most patients, and moreover the preparations are impure and unpalatable.

Certain tablets have been formulated which pass through the acid regions of the stomach and discharge the enzyme only in the relatively alkaline environment of the jejunum. However, many patients suffering from pancreatic disorders have an abnormally acid jejunum and in those cases the tablets may fail to discharge the enzyme.

Moreover, since the preparations presently on the market are of a non-human source there is a risk of immunoreactions that may cause harmful effects to the patients or result in reduced therapy efficiency. A further drawback with the present preparations is that their content of other lipolytic activities than Colipase-Dependent Lipase are not stated. In fact, most of them contain very low levels of BSSL/CEH activity. This may be one reason why many patients, suffering from cystic fibrosis in spite of supplementation therapy, suffer from deficiencies of fat soluble vitamins and essential fatty acids.

Thus, there is a great need for products with properties and structure derived from human lipases and with a broad substrate specificity, which products may be orally administered to patients suffering from deficiency of one or several of the pancreatic lipolytic enzymes. Products that can be derived from the use of the present invention fulfil this need by themselves, or in combination with preparations containing other lipases.

## SHORT DESCRIPTION OF THE INVENTIVE CONCEPT

Recombinant BSSL variants according to the invention, have maintained catalytic activity, but contain less glycosylation sites than full-length BSSL, and are thus produced with a potentially reduced degree of carbohydrate heterogeneity. This reduced complexity facilitates purification and characterization of the recombinant protein, which will result in a more cost-effective production of polypeptides having BSSL activity.

- In another aspect, the reduced degree of glycosylation is less demanding for the host and allows higher production in several host cells. In yet another aspect, the reduced number of glycosylation sites in a BSSL variant allows efficient production in lower eukaryotes and restricts the potential risk of aberrant glycosylation, which may raise immunological reactions. The reduced size and less complex glycosylation also implies that the host range is broader than for a protein having very complex and heavy carbohydrate moieties.
- Therapeutic use of a BSSL variant which is smaller in size but is equally active, means that the weight of the substance needed for supplementation is reduced. A further possible advantage with a recombinant BSSL variant lacking most or all of the O-glycosylated repeats is a reduced risk for an immunological response in the recipient individual. This is due to the fact that the O-linked sugar may be very heterogenous depending on the cell in which it is produced.
- There are indications in the scientific literature that native BSSL binds to, and is taken up by, the intestinal mucosa. A BSSL variant which is selected for having a reduced uptake, will be active on the dietary lipid substrates for a longer period of time, leading to a more efficient intraluminal digestion. Examples of such variants are molecules with reduced glycosylation.
- As mentioned above, BSSL has been suggested to be of particular importance for the utilization of long-chain polyunsaturated fatty acids (Hernell et al., 1993), which are of great importance for neuro-development of the newborn infant, and of vitamin A. A BSSL variant according to the invention, which is more effective in these respects, can be selected by known methods. A truncated, or shortened, enzyme is likely to be different with regard to conformation which may affect the specificity against different lipid substrates.



## DISCLOSURE OF THE INVENTION

5 In one aspect, the invention relates to a nucleic acid molecule encoding a polypeptide which is a BSSL variant shorter than 722 amino acids, said BSSL variant comprising part of the amino acid sequence shown as residues 536-722 in SEQ ID NO: 3.

10 The term "part of the amino acid sequence" is to be understood as comprising one single amino acid as well as a sequence of several amino acids or several such sequences combined.

15 The term "BSSL variant" is to be understood as a polypeptide having BSSL activity and comprising a part of the amino acid sequence of human BSSL shown as SEQ ID NO: 3 in the Sequence Listing.

The term "polypeptide having BSSL activity" is to be understood as a polypeptide comprising at least the properties

- 20 (a) suitable for oral administration;  
(b) activated by specific bile salts;  
(c) acting as a non-specific lipase in the contents of the small intestines, i.e. being able to hydrolyze lipids relatively independent of their chemical structure and physical state (emulsified, micellar, soluble);

25

and optionally one or more of the properties

- (d) ability to hydrolyze triacylglycerols with fatty acids of different chain-length and different degree of unsaturation;  
30 (e) ability to hydrolyze also diacylglycerol, monoacylglycerol, cholesteryl esters, lysophosphatidylacylglycerol, and retinyl and other fat soluble vitamin-esters;

- (f) ability to hydrolyze not only the sn-1(3) ester bonds in a triacylglycerol but also the sn-2 ester bond;
- (g) ability to interact with not only primary but also secondary bile salts;
- (h) dependent on bile salts for optimal activity;
- 5 (i) stable in the sense that gastric contents will not affect the catalytical efficiency to any substantial degree;
- (j) stable against inactivation by pancreatic proteases, e.g. trypsin, provided bile salts are present;
- (k) ability to bind to heparin and heparin derivatives, e.g. heparan  
10 sulphate;
- (l) ability to bind to lipid-water interphases;
- (m) stable enough to permit lyophilization;
- (n) stable when mixed with food constituents such as in human milk, or milk formula.

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In further aspects, the invention relates to a nucleic acid molecule according to above, wherein the said BSSL variant has a phenylalanine residue in its C-terminal position, or comprises the sequence Gln-Met-Pro in its C-terminal part, alternatively comprises the amino acid sequence  
20 shown as residues 712-722 in SEQ ID NO: 3 in its C-terminal part.

In the present context, the term "C-terminal position" designates the position of the final C-terminal residue, while the term "C-terminal part" is to be understood as the approximately 50 amino acid residues which  
25 constitute the C-terminal end of the BSSL variant.

The invention further relates to a nucleic acid molecule according to above, wherein the said BSSL variant comprises less than 16 repeat units. In the present context the term "repeat unit" designates one of the repeated units  
30 of 33 nucleotides each which are indicated in SEQ ID NO: 1 in the Sequence Listing.

In further aspects, the invention relates to a nucleic acid molecule according to above which encodes a polypeptide, the amino acid sequence of which is at least 90% homologous with the amino acid sequence shown as SEQ ID NO: 5, 6 or 9 in the Sequence Listing, as well as a nucleic acid molecule which encodes a polypeptide, the amino acid sequence of which is at least 90% homologous with the amino acid sequence shown as SEQ ID NO: 7 in the Sequence Listing, with the exception for those nucleic acid molecules which encode polypeptides which have an asparagine residue at position 187.

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The invention also relates to a polypeptide shown as SEQ ID NO: 5, 6, 7 or 9 in the Sequence Listing, as well as a polypeptide encoded by a nucleic acid sequence according to above.

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The invention further relates to a hybrid gene comprising a nucleic acid molecule according to above, a replicable expression vector comprising such a hybrid gene, and a cell harbouring such a hybrid gene. This cell may be a prokaryotic cell, a unicellular eukaryotic organism or a cell derived from a multicellular organism, e.g. a mammal.

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In the present context the term "hybrid gene" denotes a nucleic acid sequence comprising on the one hand a nucleic acid sequence encoding a BSSL variant as defined above and on the other hand a nucleic acid sequence of the gene which is capable of mediating the expression of the hybrid gene product. The term "gene" denotes an entire gene as well as a subsequence thereof capable of mediating and targeting the expression of the hybrid gene to the tissue of interest. Normally, said subsequence is one which at least harbours one or more of a promoter region, a transcriptional start site, 3' and 5' non-coding regions and structural sequences.

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The hybrid gene is preferably formed by inserting *in vitro* the nucleic acid sequence encoding the BSSL variant into the gene capable of mediating

expression by use of techniques known in the art. Alternatively, the nucleic acid sequence encoding the BSSL variant can be inserted *in vivo* by homologous recombination.

- 5 In the present context, the term "replicable" means that the vector is able to replicate in a given type of host cell into which it has been introduced. Immediately upstream of the nucleic acid sequence there may be provided a sequence coding for a signal peptide, the presence of which ensures secretion of the BSSL variant expressed by host cells harbouring the vector.
- 10 The signal sequence may be the one naturally associated with the nucleic acid sequence or of another origin.

The vector may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend

15 on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication; examples of such a vector are a plasmid, phage, cosmid, mini-chromosome or virus. Alternatively, the vector may be one

20 which, when introduced in a host cell, is integrated in the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable vectors are a bacterial expression vector and a yeast expression vector. The vector of the invention may carry any of the nucleic acid sequences of the invention as defined above.

- 25 In another aspect, the invention relates to a process for the production of a recombinant polypeptide, said process comprising (i) inserting a nucleic acid molecule according to above in a hybrid gene which is able to replicate in a specific host cell or organism; (ii) introducing the resulting recombinant hybrid gene into a host cell or organism; (iii) growing the
- 30 resulting cell in or on a culture medium, or identifying and reproducing an

organism, for expression of the polypeptide; and (iv) recovering the polypeptide.

5 The medium used to grow the cells may be any conventional medium suitable for the purpose. A suitable vector may be any of the vectors described above, and an appropriate host cell may be any of the cell types listed above. The methods employed to construct the vector and effect introduction thereof into the host cell may be any methods known for such purposes within the field of recombinant DNA. The recombinant human  
10 BSSL variant expressed by the cells may be secreted, i.e. exported through the cell membrane, dependent on the type of cell and the composition of the vector.

15 If the BSSL variant is produced intracellularly by the recombinant host, that is, is not secreted by the cell, it may be recovered by standard procedures comprising cell disruption by mechanical means, e.g. sonication or homogenization, or by enzymatic or chemical means followed by purification.

20 In order to be secreted, the DNA sequence encoding the BSSL variant should be preceded by a sequence coding for a signal peptide, the presence of which ensures secretion of the BSSL variant from the cells so that at least a significant proportion of the BSSL variant expressed is secreted into the culture medium and recovered.

25 The invention also relates to an expression system, comprising a hybrid gene which is expressible in a host cell or organism harbouring said hybrid gene, so that a recombinant polypeptide is produced when the hybrid gene is expressed, said hybrid gene being produced by inserting a nucleic acid  
30 sequence according above into a gene capable of mediating expression of the said hybrid gene.

A possible process for producing a recombinant BSSL variant of the invention is by use of transgenic non-human mammals capable of excreting the BSSL variant into their milk. The use of transgenic non-human mammals has the advantage that large yields of the recombinant BSSL variant are obtainable at reasonable costs and, especially when the non-human mammal is a cow, that the recombinant BSSL variant is produced in milk which is the normal constituent of, e.g., infant formulae so that no extensive purification is needed when the recombinant BSSL variant is to be used as a nutrient supplement in milk-based products.

Furthermore, production in a higher organism such as a non-human mammal normally leads to the correct processing of the mammalian protein, e.g. with respect to post-translational processing as discussed above and proper folding. Also large quantities of a substantially pure BSSL variant may be obtained.

Accordingly, the expression system referred to above may be a mammalian expression system comprising a DNA sequence encoding a BSSL variant inserted into a gene encoding a milk protein of a non-human mammal, so as to form a hybrid gene which is expressible in the mammary gland of an adult female of a mammal harbouring said hybrid gene.

The mammary gland as a tissue of expression and genes encoding milk proteins are generally considered to be particularly suitable for use in the production of heterologous proteins in transgenic non-human mammals, as milk proteins are naturally produced at high expression levels in the mammary gland. Also, milk is readily collected and available in large quantities. In the present connection, the use of milk protein genes in the production of a recombinant BSSL variant has the further advantage that it is produced under conditions similar to its natural production conditions in terms of regulation of expression and production location (the mammary gland).

When used in a transgenic mammal, the hybrid gene referred to above preferably comprises a sequence encoding a signal peptide so as to enable the hybrid gene product to be secreted correctly into the mammary gland.

5 The signal peptide will typically be the one normally found in the milk protein gene in question or one associated with the DNA sequence encoding the BSSL variant. However, also other signal sequences capable of mediating the secretion of the hybrid gene product to the mammary gland are relevant. Of course, the various elements of the hybrid gene  
10 should be fused in such a manner as to allow for correct expression and processing of the gene product. Thus, normally the DNA sequence encoding the signal peptide of choice should be precisely fused to the N-terminal part of the DNA sequence encoding the BSSL variant. In the hybrid gene, the DNA sequence encoding the BSSL variant will normally  
15 comprise its stop codon, but not its own message cleavage and polyadenylation site. Downstream of the DNA sequence encoding the BSSL variant, the mRNA processing sequences of the milk protein gene will normally be retained.

20 A number of factors are contemplated to be responsible for the actual expression level of a particular hybrid gene. The capability of the promoter as well of other regulatory sequences as mentioned above, the integration site of the expression system in the genome of the mammal, the integration site of the DNA sequence encoding the BSSL variant in the milk protein  
25 encoding gene, elements conferring post-transcriptional regulation and other similar factors may be of vital importance for the expression level obtained. On the basis of the knowledge of the various factors influencing the expression level of the hybrid gene, the person skilled in the art would know how to design an expression system useful for the present purpose.

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The milk protein gene to be used may be derived from the same species as the one in which the expression system is to be inserted, or it may be

derived from another species. In this connection it has been shown that the regulatory elements that target gene expression to the mammary gland are functional across species boundaries, which may be due to a possible common ancestor (Hennighausen et al., 1990).

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Examples of suitable genes encoding a milk protein or effective subsequences thereof to be used in the construction of an expression system of the invention, are normally found among whey proteins of various mammalian origins, e.g. a whey acidic protein (WAP) gene, preferably of murine origin, and a  $\beta$ -lactoglobulin gene, preferably of ovine origin. Also casein genes of various origins may be found to be suitable for the transgenic production of a BSSL variant, e.g. bovine  $\alpha$ S1-casein and rabbit  $\beta$ -casein. The presently preferred gene is a murine WAP gene as this has been found to be capable of providing a high level of expression of a number of foreign human proteins in milk of different transgenic animals (Hennighausen et al, 1990).

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Another sequence preferably associated with the expression system of the invention is a so-called expression stabilizing sequence capable of mediating high-level expression. Strong indications exist that such stabilizing sequences are found in the vicinity of and upstreams of milk protein genes.

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Included in the invention is also a process of producing a transgenic non-human mammal capable of expressing a BSSL variant, comprising (a) introducing an expression system according to above into a fertilized egg or a cell of an embryo of a non-human mammal so as to incorporate the expression system into the germline of the mammal and (b) developing the resulting introduced fertilized egg or embryo into an adult female non-human mammal.

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The incorporation of the expression system into the germline of the mammal may be performed using any suitable technique, e.g. as described in "Manipulating the Mouse Embryo"; A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1986. For instance, a few hundred molecules of the expression system may be directly injected into a fertilized egg, e.g. a fertilized one cell egg or a pro-nucleus thereof, or an embryo of the mammal of choice, and the microinjected eggs may then be transferred into the oviducts of pseudopregnant foster mothers and allowed to develop.

The process of producing a transgenic non-human mammal capable of expressing a BSSL variant, can also comprise a process wherein the said mammal is substantially incapable of expressing BSSL from the mammal itself. Such a process comprises (a) destroying the BSSL expressing capability of the mammal so that substantially no mammalian BSSL is expressed and inserting an expression system according to above into the germline of the mammal in such a manner that a BSSL variant is expressed in the mammal; and/or (b) replacing the mammalian BSSL gene or part thereof with an expression system as defined above.

The mammalian BSSL expressing capability can conveniently be destroyed by introduction of mutations in the DNA sequence responsible for the expression of BSSL. Such mutations may comprise mutations which make the DNA sequence out of frame, introduction of a stop codon, or a deletion of one or more nucleotides of the DNA sequence.

The mammalian BSSL gene or a part thereof may be replaced with an expression system as defined above or with a DNA sequence encoding the BSSL variant by use of the well known principles of homologous recombination.

In a further important aspect, the invention relates to a transgenic non-human mammal harbouring in its genome a DNA sequence according to

above. The said DNA sequence can preferably be present in the germline of the mammal, and in a milk protein gene of the mammal.

The transgenic non-human mammal can preferably be selected from the group consisting of mice, rats, rabbits, sheep, pigs and cattle.

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Included in the invention are also progeny of a transgenic non-human mammal according to above as well as milk obtained from such a transgenic non-human mammal.

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The invention further relates to an infant formula comprising milk according to above, and an infant formula comprising a BSSL variant as defined above. The infant formula may be prepared using conventional procedures and contain any necessary additives such as minerals, vitamins etc.

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In further aspects, the invention relates to a pharmaceutical composition comprising a BSSL variant as defined above, as well as such a BSSL variant for use in therapy.

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In yet further aspects, the invention relates to the use of a BSSL variant as defined above for the manufacture of a medicament for the treatment of a pathological condition related to exocrine pancreatic insufficiency; cystic fibrosis; chronic pancreatitis; fat malabsorption; malabsorption of fat soluble vitamins; fat malabsorption due to physiological reasons. The

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invention also relates to the use of a BSSL variant for the manufacture of a medicament for the improvement of the utilization of dietary lipids, particularly in preterm born infants.

## EXAMPLES

## 1. EXPRESSION OF RECOMBINANT BSSL IN EUKARYOTIC AND PROKARYOTIC CELLS

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## 1.1. EXPERIMENTAL PROCEDURES

## 1.1.1. Recombinant plasmids

10 The plasmid pS146 containing the 2.3 kb human BSSL cDNA (Nilsson et al., 1990) cloned into pUC19 was digested with *Hind*III and *Sal*I and the BSSL cDNA was introduced into a bovine papilloma virus (BPV) expression vector, pS147 (Fig. 1). This vector contains the human BSSL cDNA under control of the murine metallothioneine 1 (mMT-1) enhancer and promoter element (Pavlakakis & Hamer, 1983). The mRNA processing signals are provided by a genomic fragment containing part of exon II, intron II, exon III and downstream elements of the rabbit  $\beta$ -globin gene. This transcriptional unit was cloned into a vector containing the entire BPV genome. Transcription was unidirectional for BPV and the BSSL transcriptional unit. For propagation of the vector in *E.coli* the vector also contains pML2d, a pBR322 derivative (Sarver et al., 1982).

25 The expression vector pS147 was co-transfected with a vector encoding the neomycin resistance gene driven by the Harvey Sarcoma virus 5'-Long terminal repeat and Simian virus 40 polyadenylation signals (Lusky & Botchan, 1984).

30 For expression of BSSL in *E.coli*, the BSSL cDNA was subcloned as a *Nde*I-*Bam*HI fragment from plasmid pT7-7 (Ausubel et al., 1992) into plasmid pGEMEX-1 (Promega, Madison, WI, USA) (Studier & Moffat, 1986). By this cloning procedure the T7 gene 10 encoding sequence was replaced by the BSSL gene coding for the mature protein preceded by a start codon. The

final expression vector, pGEMEX/BSSL, was verified by DNA sequencing using specific BSSL internal primers.

#### 1.1.2. Mutagenesis

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Nucleotide number 1 was assigned to the A in the initiation codon ATG. For amino acid numbering the first methionine in the signal peptide is -23 and the first amino acid residue of the mature protein, an alanine, is assigned number 1.

10

For the construction of the deletion variant A (SEQ ID NO: 4), two PCR primers were synthesized, PCR-1 and PCR-2 (Table 1). The *HindIII*, *Sall* and *BamHI* sites were created for cloning into different plasmids. The *BclI* site was generated in the BSSL sequence without altering the amino acid sequence. This was done to facilitate addition of synthetic DNA to obtain the other variants. The primer PCR-2 contains two synthetic stop codons. The resulting PCR fragments were digested with *BamHI* and *HindIII* and cloned into pUC18 for sequence analysis. This plasmid was designated pS157. The correct PCR fragment was inserted into the BPV expression vector by fusion to the BSSL sequence at the unique *Asp700* site (position 1405 in the BSSL cDNA) and the *Sall* site in front of the  $\beta$ -globin gene fragment, resulting in pS257.

15

20

The B-variant construction (SEQ ID NO: 5) was done using oligonucleotides number 3,4,7 and 8 (Table 1). The annealed oligonucleotides encodes the very C-terminal amino acid sequence, representing lysine 712 to phenylalanine 722 in the full-length protein. This fragment was fused to glutamine 535. A translational stop was inserted directly after the last phenylalanine. This fragment contains a *BclI* site in the 5'-end and a *Sall* site in the 3'-end, allowing introduction into pS157. The resulting plasmid was digested with *Asp700* and *Sall* and the 313 bp

25

30

fragment was introduced into the expression vector as described above. The resulting plasmid was designated pS258.

5 TABLE 1.

Synthetic oligonucleotides used for construction of the BSSL variants.

Nucleotides of restriction sites are underlined. Translational stop signals are indicated by bold letters. The altered codon in variant N is indicated in PCR-3 by bold letters and an asterisk.

Oligo-nucleotide	Sequence (5'- 3')
PCR-1	CGGGATCCGAAGCCCTTCGCCACCCCCACG
PCR-2	CGAAGCTTGTTCGACT <b>TACTACT</b> ATGATCAGTCACTGTGGGCAGCGCCAG
PCR-3	GGGAAT <b>TCT</b> GGCCATTGCTTGGGTGAAGAGGAATATCGCGGCCTTCGG GGGGGACCCCAACC <b>AG</b> ATCACGCTCTTCGGGGAGTCT *
PCR-4	CGGGATCCACATAGTGCAGCATGGGGTACTCCAGGCC
1	<u>GATCAG</u> GGGGGCCCCCCCCGTGCCGCCACGGGTGACTCCGGG
2	GCCCCCCCCGTGCCGCCACGGGTGACTCCAAGGAAGCTCAGA
3	TGCCTGCAGTCATTAGGTTTT <b>AGTAAG</b> TCGACA
4	<u>AGCTTGTTCGACT</u> <b>TACT</b> AAAACCTAATGACTG
5	CAGGCATCTGAGCTTCCTTGGAGTCACCCGTGGGCGGCACGGGGGGGG CCCCGGA
6	GTCACCCGTGGGCGGCACGGGGGGGGCCCCCT
7	<u>GATCAGA</u> AGGAAGCTCAGA
8	CAGGCATCTGAGCTTCCTTCT

25 In order to construct the gene encoding the C-variant (SEQ ID NO: 6), oligonucleotides 1 to 6 (Table 1) were used. The annealed DNA fragment contains two repetitions, encoding eleven amino acids, identical to consensus (Nilsson et al., 1990), inserted between glutamine 535 and the

lysine 712 to phenylalanine 722 sequence. This fragment also contains a *Bcl*I site in the 5'-end and a *Sal*I site in the 3'-end, allowing the same cloning strategy as above. The resulting plasmid was designated pS259.

5 For the construction of variant N (non-N-glycosylated variant, SEQ ID NO: 7), two PCR primers (PCR-3 and PCR-4 in Table 1), were synthesized. The *Eco*RI and *Bam*HI sites were created for cloning of the 360 bp PCR product into pUC19 for sequence analysis. The potential N-linked glycosylation site at asparagine 187, was changed to a glutamine. The modified sequence  
10 was isolated as a *Bal*I-*Hind*III fragment and cloned into *Sac*I and *Hind*III digested pUC19 together with a *Sac*I and *Bal*I fragment containing the mMT-1 promoter and 5'-end of BSSL cDNA. An approximately 1.2 kb *Sac*I-*Dra*III fragment was isolated from this plasmid and inserted in the mMT-1 element and BSSL cDNA sequence, respectively, within the expression  
15 vector. The resulting plasmid was designated pS299.

#### 1.1.3. Mammalian cell culture and transfections

The vectors were co-transfected into the murine cell line C127 (ATCC CRL  
20 1616) according to the calcium-phosphate precipitation method (Graham & Van der Eb, 1973).

The C127 cells were cultured in Ham's F12-Dulbecco's Modified Eagle's medium (DMEM) (1:1) supplemented with 10% fetal calf serum. Neomycin  
25 resistant cell clones were selected with  $1.5 \text{ mg} \times \text{ml}^{-1}$  of G418 and after 10-15 days resistant cell clones were isolated from the master plates and passaged for analysis.

#### 1.1.4. Bacterial strains and culture conditions

For expression experiments the vector pGEMEX/BSSL was transformed into *E.coli* strains JM109(DE3) and BL21(DE3)pLysS. The expression  
5 experiments were carried out as described by Studier et al. (1986). After harvesting of bacteria, the cells were pelleted by centrifugation ( $5,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ ). For preparation of periplasm- and cytoplasm fractions, the pellet was resuspended in 4 ml 20 mM Tris-Cl/20% sucrose, pH 8.0, 200  $\mu\text{l}$  0.1 M EDTA and 40  $\mu\text{l}$  lysozyme (15 mg/ml in water) per gram of  
10 pellet. The suspension was incubated on ice for 40 minutes. 160  $\mu\text{l}$  0.5 M  $\text{MgCl}_2$  per gram of pellet was added, whereafter the suspension was centrifuged for 20 min at  $12,000 \times g$ . The resulting supernatant contains periplasmic proteins and the pellet represents the cytoplasmic fraction. Alternatively, for preparation of soluble proteins, the cells were suspended  
15 in 40 mM Tris-Cl, 0.1 mM EDTA, 0.5 mM phenylmethylsulphonylfluoride, pH 8.2, freeze-thawed and sonicated several times to lyse. The cell lysate was centrifuged ( $30,000 \times g$  for 30 min at  $25^{\circ}\text{C}$ ).

#### 1.1.5. Nucleic acid analysis

20 RNA and DNA were prepared from isolated mammalian cell lines or *E.coli* cells (Ausubel et al., 1992). The RNA or DNA were fractionated on agarose gels and blotted onto GeneScreen Plus (New England Nuclear) and hybridized according to the supplier's instructions.

25

#### 1.1.6. Preparation of native enzyme

Bile salt-stimulated lipase was purified from human milk as previously described (Bläckberg & Hernell, 1981). The purified preparation was  
30 homogenous as judged by SDS-PAGE and had a specific activity of 100  $\mu\text{mol}$  fatty acid released  $\times \text{min}^{-1}$  and  $\text{mg}^{-1}$  when assayed with long-chain triacylglycerol as substrate.

#### 1.1.7. Enzyme assay

The enzyme assay was as described (Bläckberg & Hernell, 1981) using triolein emulsified with gum arabic as substrate. The incubations were carried out with 10 mM sodium cholate as activating bile salt. When the bile salt dependency was tested bile salts (sodium cholate or sodium deoxycholate, Sigma Chem. Co.) were added to the concentrations given in Fig. 3.

#### 1.1.8. Western blotting

In order to obtain significant reactions in the blotting experiments the conditioned media were concentrated by chromatography on Blue Sepharose (Pharmacia LKB Biotechnology). The respective media were mixed with Blue Sepharose (approx 10 ml of medium per ml of gel). The gel was washed with (10 ml per ml of gel) with 0.5 M Tris-Cl buffer, pH 7.4, containing 0.1 M KCl. The enzyme activity was eluted with 1.5 M KCl in the same buffer. By this procedure a 25-30-fold concentration was obtained as well as a 3-5-fold purification. SDS-PAGE was performed on 10% polyacrylamide gels essentially according to Laemmli (1970). After transfer to nitrocellulose membranes and incubation with a polyclonal rabbit antiserum to purified BSSL detection was made using goat anti-rabbit IgG conjugated with alkaline phosphatase and a developing kit from Bio-Rad.

#### 1.1.9. Treatment with N-glycosidase F

To 10  $\mu$ l of variant B, containing a BSSL activity of 2.5  $\mu$ mol fatty acid released  $\times$  min<sup>-1</sup>, 1  $\mu$ l of 1 M  $\beta$ -mercaptoethanol and 0.5  $\mu$ l of 10% (w/v) SDS was added. After boiling for 5 min, 10  $\mu$ l 0.1 M Na-phosphate buffer, pH 8.0, 6  $\mu$ l 0.1 M EDTA, 4  $\mu$ l 7.5% (w/v) Nonidet P 40 and 5  $\mu$ l (1U) N-glycosidase F (Boehringer Mannheim) were added. As a control the same



amount of variant B was treated identically except that no glycosidase was added. After an overnight incubation at 37°C, the samples were run on SDS-PAGE and blotted using the polyclonal rabbit BSSL antiserum.

## 5 1.2. RESULTS

### 1.2.1. Construction of the BSSL variants

10 The modifications of the BSSL variants in relation to the full-length BSSL are summarized in Table 2 and Fig. 1. The strategies used for generation of these variants are described in Section 1.1. For variant A (SEQ ID NO: 4), a stop codon was introduced after glutamine at position 535 thereby removing the last 187 amino acids of the full-length protein. For variant B (SEQ ID NO: 5) the domain encoding the 11 very C-terminal amino acids and the original translational stop was fused to glutamine-535. Hence, this  
15 variant lacks all the repeats. For variant C (SEQ ID NO: 6) a fragment containing two repeats having a sequence identical to consensus (Nilsson et al., 1990) were inserted between glutamine-535 and the lysine-712 to phenylalanine-722 sequence.

20

To analyze the importance of the only tentative N-linked carbohydrate structure, positioned close to the active site serine-194, a variant was constructed. Variant N (SEQ ID NO: 7) was obtained by altering the potential N-glycosylation site at asparagine-187 to a glutamine.

25

TABLE 2

The amino acid sequence of the BSSL variants in relation to that of human BSSL.

Variant	Deleted residues	Changed residues
A (SEQ ID NO: 4)	536-722	
B (SEQ ID NO: 5)	536-711	
C (SEQ ID NO: 6)	536-568, 591-711	
N (SEQ ID NO: 7)		Asn 187 → Gln

#### 1.2.2. Characterization of recombinant DNA in the mammalian cell lines

DNA samples were prepared from the cell lines transfected with the expression vectors encoding the different BSSL variants. The prepared DNA was digested with *Bam*HI, fractionated on agarose gels and transferred to membranes for hybridization. The probe used was <sup>32</sup>P-labelled BSSL cDNA. The hybridization results confirmed the presence of the recombinant genes and also that the vector copy number was approximately equal in the different cell lines (Fig. 2). The positions of the hybridizing fragments reflected the different lengths of the various BSSL sequences and were in agreement with the expected sizes. The positions were also similar to the bacteria derived DNA used in the transfection experiment, indicating that no major rearrangement of vector DNA had occurred in the cell lines (Fig. 2). The upper hybridization signals in the DNA sample representing variant A were probably due to partial digestion.

### 1.2.3. Expression of mRNA for full-length and mutated BSSL in mammalian cells

To analyze the expression of the different recombinant BSSL genes RNA was prepared from the isolated cell lines. Northern blot experiments and hybridization with  $^{32}\text{P}$ -labelled BSSL cDNA showed that recombinant mRNA was detectable in all cell lines harboring a BSSL vector (Fig. 3). No hybridization was found in the control sample derived from a cell line containing an identical vector except for BSSL cDNA (Fig. 3).

The different lengths of the hybridizing mRNAs were in accordance with the modifications of the cDNAs. The steady state levels of recombinant BSSL mRNA variants in the different samples were about the same except for variant A (Fig. 3). The reason for the reduced accumulation of variant A mRNA is not known, but it was observed with two populations of cell lines as well as with isolated clones. The presence of equal amounts of RNA in the different samples was confirmed by hybridization to a murine  $\beta$ -actin probe (Fig. 3, lower panel).

### 1.2.4. Production of full-length and variants of BSSL in mammalian cells

Media from individual clones of the C127-cells, transfected with full-length BSSL and the different mutated forms, were collected and assayed for BSSL activity (Fig. 4). For the full-length molecule and variants N, B and C the activities in the clones with the highest expression ranged from 0.7 to 2.3  $\mu\text{mol}$  fatty acid released  $\times \text{min}^{-1} \times \text{ml}$  of medium $^{-1}$ . With a specific activity comparable to that of the native milk BSSL this would correspond to expression levels of 7-23  $\mu\text{g}$   $\times \text{ml}$  medium $^{-1}$ . For variant A all the analyzed clones had activities below 0.05  $\mu\text{mol}$  fatty acid released  $\times \text{min}^{-1}$  and  $\text{ml}$  of medium $^{-1}$ . Concentration on Blue-Sepharose and lyophilization of the clone showing the highest activity revealed that an active enzyme indeed was expressed, albeit at very low levels. The possibility that the low

activity obtained with variant A in part could be explained by a considerably lower specific activity could not be ruled out.

Western blots from clones of the different transfection experiments are shown in Fig. 5A. The apparent  $M_r$  of the BSSL variants were as expected. It should be noted, however, that for full-length BSSL as well as for variants B and C a double band was obtained. Because all three have the single N-glycosylation site intact whereas variant N, which showed no double band, lacks that site, a likely explanation was that the double band resulted from differences in N-glycosylation. Therefore variant B was subjected to digestion with N-glycosidase F. As shown in Fig. 5B, only trace amounts of the upper band remained while the lower band increased in strength indicating that only part of the expressed variant was N-glycosylated.

One of the characteristics of BSSL is its specific activation by primary bile salts, e.g. cholate (Hernell, 1975). All the different recombinant forms of BSSL showed the same concentration dependency for cholate activation (Fig. 6). A maximal activity was obtained at about 10 mM in the assay system used. When cholate was exchanged for deoxycholate (a secondary bile salt) no such activation occurred. Thus, the recombinant full-length as well as the different variants showed the same specificity regarding bile salt activation.

#### 1.2.5. Expression and biochemical characterization of full-length BSSL in *E.coli*

Two *E.coli* strains JM109(DE3) and BL21(DE3)pLysS (Studier et al., 1986) were transformed with the expression vector pGEMEX/BSSL containing the human BSSL cDNA under control of the T7 promoter. Transformants from both strains were identified, cultured and induced with IPTG for about 90 min (Studier et al., 1986). Analysis of total mRNA by Northern

- blot using the BSSL cDNA as a  $^{32}\text{P}$ -labeled probe demonstrated that expression was efficiently induced in both strains and that the transcription was tightly regulated (Fig. 7A). The apparent size of the recombinant BSSL mRNA, approximately 2.4 kb, is in agreement with the expected length.
- 5 SDS-PAGE separation of protein samples and immunodetection with anti-BSSL antibodies showed that full-length BSSL was efficiently produced in *E.coli* (Fig. 7B). More of the protein was secreted to the periplasm in the BL21(DE3)pLysS strain than in JM109(DE3) (Fig 7B).
- 10 IPTG-induced *E.coli* cultures contained active soluble BSSL corresponding to 0.5 - 4  $\mu\text{g}$  of BSSL protein/ml culture. Western blotting showed that between 20 and 60% of the reactive material was in the insoluble pellet. Uninduced bacteria did not contain any significant BSSL activity.
- 15 The lipase activity from cultured bacteria showed the same bile salt dependence as native milk BSSL.

## 20 2. PURIFICATION AND CHARACTERIZATION OF RECOMBINANT FULL-LENGTH AND MUTATED FORMS OF BILE SALT-STIMULATED LIPASE

### 2.1. EXPERIMENTAL PROCEDURES

#### 25 2.1.1. Enzymes and enzyme variants

Recombinant full-length BSSL and BSSL variants B, C and N were constructed and expressed as previously described. Compared to the native enzyme Variant B (SEQ ID NO: 5) lacks all 16 unique, O-glycosylated, proline-rich, C-terminal repeats (aa 536-711) but with the most C-terminal  
30 fragment (aa 712-722) fused to glutamine-535. Variant C (SEQ ID NO: 6) contains the same C-terminal fragment and two repeats of 11 residues between glutamine-535 and lysine-712. In variant N (non-N-glycosylated

variant, SEQ ID NO: 7) the asparagine-187 responsible for the only N-linked sugar was exchanged for a glutamine residue.

Native BSSL was purified from human milk as described (Bläckberg & Hernell, 1981).

5

#### 2.1.2. Enzyme assay

Lipase activity was assayed as described (Bläckberg & Hernell, 1981) using triolein emulsified in gum arabic as substrate. Sodium cholate (10 mM)  
10 was used as activating bile salt. Different modifications of the assay are given in legends to figures.

#### 2.1.3. Preparation of immunosorbent

15 Purified milk BSSL (5 mg) was coupled to Sepharose using CNBr as described by the manufacturer. 40 ml of a polyclonal antiserum raised in rabbit against purified milk BSSL was passed over the column. Specific antibodies were eluted with 0.1 M glycine-HCl, pH 2.5. The pH was immediately adjusted to approx 8 with solid Tris. After desalting and  
20 lyophilization 6 mg of the affinity purified antibodies was coupled to Sepharose as described above.

#### 2.1.4. Purification procedure

25 Conditioned culture media containing 5-25 µg of recombinant expressed BSSL or BSSL variant was mixed Blue Sepharose (Pharmacia, Sweden) 10 ml media per ml of settled gel. After end-to-end mixing for 30 min the gel was rinsed with 0.05 M Tris-Cl, pH 7.0, 0.05 M KCl and the lipase activity eluted with 0.05 M Tris-Cl, pH 7.0, 1.5 M KCl. The activity peak was  
30 pooled and dialyzed against 5 mM sodium veronal, pH 7.4, 0.05 M NaCl. The dialyzate was applied to a heparin-Sepharose column. The column was eluted with a gradient 0.05 to 1.0 M NaCl in 5 mM sodium veronal buffer,

pH 7.4. Fractions containing lipase activity were pooled and applied to an immunosorbent column. After rinsing with 0.05 M Tris-Cl, pH 7.5, 0.15 M NaCl lipase bound was eluted with 0.1 M glycine-HCl, pH 2.5. The pH of the fractions was immediately adjusted to approx 8 with solid Tris.

5

#### 2.1.5. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli (1970). Proteins were stained with Commassie Brilliant Blue.

10

#### 2.1.6. N-terminal sequence analysis

Amino acid sequence analysis were performed on an Applied Biosystems Inc. 477A pulsed liquid-phase sequencer and an on-line phenylthiohydantoin 120A analyzer with regular cycle programs and chemicals from the manufacturer. Calculated from a sequenced standard protein ( $\beta$ -lactoglobulin) initial and repetitive yields were 47% and 97%, respectively.

15

20

### 2.2. RESULTS

#### 2.2.1. Purification of recombinant BSSL and BSSL variants.

Chromatography on Blue Sepharose of conditioned media was primarily used to as a concentrating step. The subsequent chromatography on heparin-Sepharose gave an initial purification mainly by removing most of the albumin present in the culture medium. This step also showed that the recombinant BSSL molecules all retained the heparin binding. After the immunosorbent all BSSL variants appeared more than 90% pure, as judged by SDS-PAGE (Fig. 8). The full-length enzyme as well as variant B and C migrated as a doublet. The apparent  $M_r$  of the different variants are shown

25

30

in Table 3. N-terminal sequence analysis gave a single sequence for all variants for 8 cycles: Ala-Lys-Leu-Gly-Ala-Val-Tyr-Thr-.

### 2.2.2. Lipase activity

5

In Table 3 the apparent molecular weight of the different preparations is shown. The specific activities of the preparations ranged from 75 to 120  $\mu\text{mol}$  free fatty acid released per min and mg protein. Consequently no significant difference in activity between full-length BSSL and the BSSL variants could be observed.

10

The preparations all showed an absolute requirement for primary bile salt (sodium cholate) for activity against emulsified long-chain triacylglycerol (Fig. 9A). Sodium deoxycholate did render any of the variants active (data not shown). However, when combining the different bile salts deoxycholate had two effects (Fig. 9B and C). Firstly, it lowered the concentration of cholate needed for activation, and secondly it inhibited enzyme activity at higher bile salt concentration.

15

20

TABLE 3.

Apparent  $M_r$  of recombinant full-length BSSL and BSSL variants.

Enzyme	$M_r$ (kDa) Determined by SDS-PAGE
Full-length	105, 107
Variant B	63, 65
Variant C	60, 62
Variant N	95

25



### 2.2.3. Stability of recombinant BSSL and BSSL variants

Recombinant BSSL as well as the BSSL variants showed the same pH-stability as native milk BSSL (Fig. 10). An inactivation occurred in all cases at a pH around 2.5-3. Above pH 3 all variants were completely stable provided the protein concentration was high enough. This was accomplished by adding bovine serum albumin or ovalbumin (data not shown). Diluted samples were less stable at all tested pH but the threshold remained the same (data not shown). Fig. 11 shows the heat stability of the recombinant enzymes compared to the native milk enzyme. At a temperature of 37-40°C the activity starts to decrease. The variants (B, C, N) appears to be somewhat less stable than the full-length recombinant enzyme and the milk enzyme. However, if the protein concentration was raised by adding bovine serum albumin all variants was stable also at 40°C (Fig. 11).

Native milk BSSL and all the recombinant variants were all sensitive to trypsin. A time dependent inactivation was obtained (Fig. 12). If, however, bile salts, i.e. cholate, was included in the buffer the lipase variants were protected and lipase activity retained (Fig. 12).

Thus, with regard to a number of *in vitro* characteristics, i.e. bile salt activation, heparin binding, pH- and temperature stability and bile salt protection against inactivation by proteases, no significant differences were observed when comparing the different BSSL variants with native milk BSSL.

### 3. EXPRESSION IN TRANSGENIC ANIMALS

#### 3.1. CONSTRUCTION OF EXPRESSION VECTORS

5 To construct an expression vector for production of recombinant human BSSL variant in milk from transgenic animals, the following strategy was employed (Fig.13).

10 Three plasmids containing different parts of the human BSSL gene (pS309, pS310 and pS311) were obtained using the methods described in Lidberg et al. (1992). The plasmid pS309 contains a *SphI* fragment covering the BSSL gene from the 5' untranscribed region to part of the fourth intron. The plasmid pS310 contains a *SacI* fragment covering a BSSL variant gene sequence from part of the first intron to a part of the sixth intron. The  
15 plasmid pS311, finally, contains a *BamHI* fragment covering the BSSL gene from a major part of the fifth intron and the rest of the intron/exon structure with deletions in exon 11. The deleted sequences are 231 bp which results in a sequence encoding a BSSL variant which has exactly 77 amino acids or seven repeats less than the full-length BSSL. The nucleotide  
20 sequence of the resulting BSSL variant ("Variant T") is shown in the Sequence Listing as SEQ ID NO: 8. The amino acid sequence of variant T is shown in the Sequence Listing as SEQ ID NO: 9.

25 Due to the highly repetitive sequence in exon 11 of the human BSSL gene, relatively high frequencies of rearrangements can be anticipated when this sequence is cloned into a plasmid and propagated in bacteria. Based on this assumption, one desired BSSL variant which contains a truncated exon 11, was identified, isolated and subjected to sequence analysis.

30 Another plasmid, pS283, containing a part of the human BSSL cDNA cloned into the plasmid pUC19 at the *HindIII* and *SacI* sites was used for fusion of the genomic sequences. Plasmid pS283 was also used to get a

proper restriction enzyme site, *KpnI*, located in the 5' untranslated leader sequence of BSSL.

5 Plasmid pS283 was digested with *NcoI* and *SacI* and a fragment of about 2.7 kb was isolated by electrophoresis. Plasmid pS309 was digested with *NcoI* and *BspEI* and a fragment of about 2.3 kb containing the 5'-part of the BSSL gene was isolated. Plasmid pS310 was digested with *BspEI* and *SacI* and a fragment of about 2.7 kb containing a part of the middle region of the BSSL gene was isolated. These three fragments were ligated and  
10 transformed into competent *E. coli*, strain TG2, and transformants were isolated by ampicillin selection.

Plasmids were prepared from a number of transformants, and one plasmid, called pS312 (Fig. 14), containing the desired construct was used  
15 for further experiments.

To obtain a modification of pS311 in which the *BamHI* site located downstream of the stop codon was converted to a *SalI* site to facilitate further cloning, the following method was used: Plasmid pS311 was  
20 linearized by partial *BamHI* digestion. The linearized fragment was isolated and a synthetic DNA linker that converts *BamHI* to a *SalI* site (5'-GATCGTCGAC-3'), thereby destroying the *BamHI* site, was inserted. Since there were two potential positions for integration of the synthetic linker the resulting plasmids were analyzed by restriction enzyme cleavage. A  
25 plasmid with the linker inserted at the desired position downstream of exon 11 was isolated and designated pS313.

To obtain the final expression vector construct harbouring the human BSSL variant genomic sequences an existing expression vector, pS314, designed  
30 to mediate stage and tissue specific expression in the mammary gland cells under lactation periods was used. Plasmid pS314 contains a genomic fragment from the murine whey acidic protein (WAP) gene (Campbell et

al., 1984) cloned as a *NotI* fragment. The genomic fragment has approximately 4.5 kb upstream regulatory sequences (URS) all the four murine WAP exons and all intron sequences and about 3 kb of sequence downstream of the last exon. A unique *KpnI* site is located in the first exon  
5 24 bp upstream of the natural WAP translation initiation codon. Another unique restriction enzyme site is the *SalI* site located in exon 3.

The human BSSL variant genomic sequence was inserted between these sites, *KpnI* and *SalI*, by the following strategy: First, pS314 was digested  
10 with *KpnI* and *SalI* and a fragment representing the cleaved plasmid was electrophoretically isolated. Second, pS312 was digested with *KpnI* and *BamHI* and a approximately 4.7 kb fragment representing the 5'-part of the human BSSL gene was isolated. Third, pS313 was digested with *BamHI* and *SalI* and the 3'-part of the human BSSL gene was isolated. These three  
15 fragments were ligated, transformed into competent *E. coli* bacteria and transformants were isolated after ampicillin selection.

Plasmids were prepared from several transformants and carefully analyzed by restriction enzyme mapping and sequence analysis. One plasmid  
20 representing the desired expression vector was defined and designated pS317 (Fig.15).

In order to remove the prokaryotic plasmid sequences, pS317 was digested with *NotI*. The recombinant vector element consisting of murine WAP  
25 sequence flanking the human BSSL variant genomic fragment was then isolated by agarose electrophoresis. The isolated fragment was further purified using electroelution, before it was injected into mouse embryos.

The recombinant gene for expression of human BSSL variant in milk from  
30 transgenic mice is shown in Figure 16.

### 3.2. GENERATION OF TRANSGENIC ANIMALS

A *NotI* fragment was isolated from the plasmid pS317 according to section 3.1. This DNA fragment contained the murine WAP promoter linked to a genomic sequence encoding human BSSL variant. The isolated fragment, at a concentration of 3 ng/ $\mu$ l, was injected into the pronucleus of 350 C57B1/6JxCBA/2J-f<sub>2</sub> embryos obtained from donor mice primed with 5 IU pregnant mare's serum gonadotropin for superovulation. The C57B1/6JxCBA/2J-f<sub>1</sub> animals were obtained from Bomholtgård Breeding and Research Centre LTD, Ry, Denmark. After collection of the embryos from the oviducts, they were separated from the cumulus cells by treatment with hyaluronidase in the medium M2 (Hogan et al., 1986). After washing the embryos were transferred to the medium M16 (Hogan et al., 1986) and kept in an incubator with 5% CO<sub>2</sub>-atmosphere. The injections were performed in a microdrop of M2 under light paraffin oil using Narishigi hydraulic micromanipulators and a Nikon inverted microscope equipped with Nomarski optics. After injection, 267 healthy looking embryos were implanted into 12 pseudopregnant C57B1/6JxCBA/2J-f<sub>1</sub> recipients given 0.37 ml of 2.5% Avertin intraperitoneally. Mice that had integrated the transgene were identified with PCR analysis of DNA from tail biopsy specimens obtained three weeks after birth of the animals. Positive results were confirmed with Southern blot analysis.

For milk collection, female lactating animals were injected with 2 IU oxytocin intraperitoneally and 10 minutes later anaesthetized with 0.40 ml of 2.5% Avertin intraperitoneally. A milk collecting device was attached to the nipple via a siliconized tubing and milk was collected into a 1.5 ml Eppendorf tube by gentle massage of the mammary gland. The amount of milk varied, dependent on the day of lactation, between 0.1 and 0.5 ml per mouse and collection.

### 3.3. EXPRESSION OF BSSL VARIANT IN TRANSGENIC MICE

Transgenic mice were identified by analysis of DNA which has been prepared from excised tail samples. The tissue samples were incubated with proteinase K and phenol/chloroform extracted. The isolated DNA was used in polymerase chain reactions with primers which amplify specific fragments if the heterologous introduced DNA representing the expression vector fragment is present. The animals were also analyzed by DNA hybridization experiments to confirm PCR data and to test for possible rearrangements, structure of the integrated vector elements and to obtain information about the copy number of integrated vector elements.

In one set of experiments, 31 mice were analyzed with the two methods and the results demonstrated that 1 mice was carrying the heterologous DNA vector element derived from pS317. The result from the PCR analysis and the hybridization experiments were identical (Fig. 17). In total, 10 of 65 tested animals were found to be transgenic for pS317.

The mouse identified to carry vector DNA element (founder animal) was then mated and the F1 litter was analyzed for transgene by the same procedures.

RNA isolated from various tissues of pS317 transgenic females during lactation have been separated by agarose formaldehyde gel electrophoresis, blotted to membranes and hybridized with  $^{32}\text{P}$ -labelled BSSL cDNA as a probe. The obtained results show that the expression is restricted to the mammary gland during lactation (Fig. 18).

Milk samples were collected from the anesthetized founder animal treated with oxytocin to induce lactation and analyzed for the presence of recombinant human BSSL variant. This was done by SDS-PAGE, transfer to nitrocellulose membranes and incubation with polyclonal antibodies

generated against native human BSSL. The obtained results demonstrated expression of recombinant human BSSL variant in milk from transgenic mice. Figure 19 demonstrates presence of recombinant human BSSL variant in milk from transgenic mice. SDS-PAGE separation and immunoblotting of milk samples derived from various pS317 transgenic mice show efficient production of a recombinant BSSL variant with reduced apparent molecular weight in comparison to full-length recombinant BSSL derived from milk of a mouse transgenic for pS314. The plasmid pS314 is similar to pS317, with the exception that pS314 contains full-length human BSSL cDNA instead of the genomic variant. The doublet band which is apparent in all murine milk samples is representing murine BSSL, and thus shows the cross reactivity of the antiserum. This conclusion is further supported by the observation that this doublet band is apparent in lane 9 of Figure 19, which contains purified murine BSSL.

Stable lines of transgenic animals are generated.

In a similar manner, other transgenic animals such as rabbits, cows or sheep capable of expressing human BSSL variants may be prepared.

## DEPOSITS

The following plasmids have been deposited in accordance with the Budapest Treaty at DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen):

5	Plasmid	Deposit No.	Date of deposit
	pS309	DSM 7101	12 June 1992
	pS310	DSM 7102	
	pS311	DSM 7103	
	pS317	DSM 7104	
10	pS147	DSM 7495	26 February 1993
	pS257	DSM 7496	
	pS299	DSM 7497	
	pS258	DSM 7501	3 March 1993
15	pS259	DSM 7502	



## BRIEF DESCRIPTION OF DRAWINGS

Figure 1

- 5 A. Map of the BPV based vector used for expression of the different BSSL variants.
- B. A schematic representation of the different BSSL variants analyzed. FL denotes the full-length BSSL. The active site is indicated by a circle and the site for the potential N-linked carbohydrate is indicated by a triangle. The region containing the repeats is indicated as a striped area and the
- 10 conserved C-terminal as a filled area.

Figure 2

- Southern blot analysis of DNA from cell lines expressing BSSL variants. DNA prepared from cell lines expressing full-length BSSL (FL), variant A (A), variant B (B), variant C (C) and variant N (N) were analyzed. 5 µg of
- 15 the respective prepared cell derived DNA (left) and 1 ng of purified bacteria derived vector DNA (right), were digested with *Bam*HI. The DNA samples were separated on an agarose gel, transferred to GeneScreen Plus membrane and hybridized with <sup>32</sup>P-labelled human BSSL cDNA.

20

Figure 3

- Northern blot analysis of RNA from isolated cell lines expressing recombinant BSSL variants. 10 µg of total RNA prepared from cell lines producing full-length BSSL (FL), variant A (A), variant B (B), variant C (C),
- 25 variant N (N) were analyzed. RNA from a C127 cell line harboring a BPV-vector identical to the vector in Fig. 1, except for that it encodes a protein unrelated to BSSL, was used as negative control (-) (upper panel). Filters were hybridized with <sup>32</sup>P-labelled BSSL cDNA. The filter was then rehybridized with a murine β-actin cDNA probe. The β-actin mRNA
- 30 signals (lower panels) were used as an internal control for the amounts of RNA loaded onto each lane.

Figure 4

Expression of BSSL activity in C127 cells transfected with full-length and mutated forms of human BSSL. C127 cells were transfected with different BSSL-constructs: full-length BSSL (FL), variant N (N), variant C (C), variant B (B), variant A (A). After the initial growth period individual clones were selected and allowed to grow until confluency. The number of selected clones (n) are indicated in the figure. Lipase activity was determined on the conditioned media. Values are expressed as  $\mu\text{mol}$  free fatty acid released  $\times \text{min}^{-1} \times \text{ml}$  of conditioned medium $^{-1}$ .

Figure 5

A. Western blotting of full-length and mutated recombinant BSSL. The amounts of lipase activity, expressed as  $\mu\text{mol}$  fatty acid released  $\times \text{min}^{-1}$ , applied to the gel was: Full-length 0.2 (lane 1), variant N 0.16 (lane 2), variant C 0.6 (lane 3), variant B 0.8 (lane 4) and native BSSL 0.1 (lane 5). The antiserum used was raised in rabbit against BSSL purified from human milk. The position of size markers (Prestained SDS-PAGE Standards, Low Range, BioRad) are indicated to the left.

B. Western blot of N-glycosidase F treated variant B. Variant B was digested with N-glycosidase F as described in Experimental procedures. Lane 1 shows untreated and lane 2 treated variant B.

Figure 6

Bile salt-dependency of full-length and mutated BSSL. Lipase activity was determined in the presence of varying concentrations of sodium cholate (solid lines) or sodium deoxycholate (broken lines) on conditioned media from full-length recombinant BSSL (\*), variant A ( $\square$ ), variant B ( $\blacktriangle$ ), variant C ( $\blacksquare$ ), variant N ( $\bullet$ ) and purified human milk BSSL (O). For the A variant conditioned medium was concentrated on Blue Sepharose as described under Experimental procedures. The amount of the respective enzyme source was chosen to obtain the same level of maximal activity except for

variant A which had a maximal activity of only one-tenth of the others. Control experiments showed that the growth media did not influence the level of activity or the bile salt dependency of native BSSL (data not shown).

5

### Figure 7

A. Northern blot of BSSL produced by different strains of *E.coli* using pGEMEX. The bacteria were induced by IPTG as described in experimental procedures.

10 Experimental conditions were as described in the legend to Figure 2. Lane 1, strain BL21(DE3)pLysS, not induced; Lane 2, strain BL21(DE3)pLysS, induced; Lane 3, strain JM109(DE3), not induced; Lane 4, strain JM109(DE3), induced.

15 B. Western blot, using antibodies to purified milk BSSL, of an 8-18% SDS-PAGE showing the expression of recombinant BSSL in different strains of *E.coli* using pGEMEX. Bacteria were induced with IPTG, and cytoplasmic and periplasmic proteins prepared from lysate as described in experimental procedures. The amounts of bacterial proteins loaded in lane 2-5  
20 (periplasmic preparations) and 7-10 (cytoplasmic preparations) represent the same culture volume making the stain proportional to the production level. Lane 1, Pharmacia molecular size markers; Lanes 2 and 8, strain JM109(DE3), induced; Lanes 3 and 7, strain JM109(DE3), not induced; Lanes 4 and 10, strain BL21(DE3)pLysS, induced; Lanes 5 and 9, strain  
25 BL21(DE3)pLysS, not induced; Lane 6, 25 ng of purified native milk BSSL.

### Figure 8

SDS-PAGE of purified recombinant BSSL and BSSL variants. Full-length recombinant BSSL (FL) and BSSL variants N, B, and C were purified as  
30 described. 3 µg of each was applied, except for variant B, of which 1.5 µg was used. 5 µg of purified native milk BSSL (NAT) was applied. The position of size markers are indicated to the left.

Figure 9

Effect of sodium deoxycholate on the activation of recombinant BSSL and BSSL variants by sodium cholate. Purified preparations of recombinant full-length BSSL (●), recombinant BSSL variants B (○), C (■) and N (▲),  
5 and purified native milk BSSL (□) were assayed for lipase activity with different concentrations of sodium cholate in the absence (left panel) and in the presence of 5 mM (centre panel) or 10 mM (right panel) deoxycholate.

Figure 10

10 Stability of recombinant BSSL and BSSL variants at different pH. Native BSSL, recombinant full-length BSSL and BSSL variants were incubated at 37°C in different buffers with pH 2-8. All buffers contained 1 mg/ml of bovine serum albumin. After 30 min aliquotes were withdrawn and assayed for lipase activity. For explanation of symbols, see the legend to  
15 Fig. 9.

Figure 11

Heat stability of recombinant BSSL and BSSL variants. Purified recombinant full-length BSSL, BSSL variants and native milk BSSL were  
20 incubated at the temperatures indicated in 50 mM Tris-Cl buffer, pH 7.5. To one set of samples bovine serum albumin (BSA) was added to 1 mg/ml. After 30 min samples were withdrawn and assayed for lipase activity. Activities are expressed as per cent of the activity for each sample at 0 min. For explanation of symbols, see the legend to Fig. 9.

25

Figure 12

Effect of bile salts on the inactivation of recombinant BSSL and BSSL variants by trypsin. Purified recombinant full-length BSSL, BSSL variants and native milk BSSL (15 µl containing 1-4 µg) were added to 60 µl of 1.0  
30 M Tris-Cl, pH 7.4 with 10 µg of trypsin (TPCK-trypsin, Boehringer-Mannheim) at 25°C in the absence (broken lines) and in the presence (solid lines) of 10 mM sodium cholate. At the times indicated aliquotes were

withdrawn and assayed for lipase activity. Values are expressed as per cent of values obtained in control incubations in the absence of trypsin. For explanation of symbols, see the legend to Fig. 9.

5     Figure 13

Method for production of the plasmid pS317. For further details, see section 3.1.

10    Figure 14

Schematic structure of the plasmid pS312.

15    Figure 15

Schematic structure of the plasmid pS317.

20    Figure 16

Physical map representing the physical introduction of human BSSL variant genomic structure in the first exon of the WAP gene as described in section 3.1.

25    Figure 17

A.     Schematic representation of the localization of PCR-primers used for identification of transgenic animals. The 5'-primer is positioned within the WAP sequence starting at the position -148 bp upstream of the fusion between the WAP and BSSL variant. The 3'-primer is localized in the first BSSL variant intron ending 400 bp downstream of the fusion point.

B.     The sequences of the PCR primers used.

C.     Agarose gel showing a typical analysis of the PCR analysis of the potential founder animals. M: molecular weight markers. Lane 1: control PCR-product generated from the plasmid pS317. Lanes 2-13: PCR reactions done with DNA preparations from potential founder animals.

Figure 18

Northern blot analysis of RNA prepared from various tissues isolated from a female mouse transgenic for pS317. The tissues were isolated at day four of lactation. 10 µg of total RNA from each tissue was analyzed by agarose-formaldehyde separation, transferred to membranes and hybridized with <sup>32</sup>P-labelled human BSSL cDNA. The lanes contain Mg: mammary gland; Li: liver; Ki: kidney; Sp: spleen; He: heart; Lu: lung; Sg: salivary gland; Br: brain. RNA sizes in nucleotides are indicated to the left.

10 Figure 19

Western blotting of milk obtained from pS317 transgenic mice, and mice transgenic for a full-length cDNA vector pS314 and control animals. The samples were separated by SDS-PAGE and transferred to Immobilon filters and immunoblotted with antiserum raised against native human BSSL.

15 Lane 1: molecular weight markers; Lanes 2,3 and 4: 2 µl milk from three F1 daughters (F1 30, 31, and 33) of pS317 founder F0 #91; Lane 5: 2 µl milk from pS314 founder #90. Lanes 6, 7 and 8: 2 µl milk from three non-BSSL transgenic animals; Lane 9: purified murine BSSL; Lane 10: purified human native BSSL.

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  - (I) TELEX: 19237 astra s
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- (iv) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: SE 9300686-4
  - (B) FILING DATE: 01-MAR-1993
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: SE 9300722-7
  - (B) FILING DATE: 04-MAR-1993

## (2) INFORMATION FOR SEQ ID NO: 1:

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  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA to mRNA
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
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			375					380					385			
GAG	ACC	GAT	GTC	CTC	TTC	CTG	GTG	CCC	ACC	GAG	ATT	GCC	CTA	GCC	CAG	1359
Glu	Thr	Asp	Val	Leu	Phe	Leu	Val	Pro	Thr	Glu	Ile	Ala	Leu	Ala	Gln	
		390					395					400				
CAC	AGA	GCC	AAT	GCC	AAG	AGT	GCC	AAG	ACC	TAC	GCC	TAC	CTG	TTT	TCC	1407
His	Arg	Ala	Asn	Ala	Lys	Ser	Ala	Lys	Thr	Tyr	Ala	Tyr	Leu	Phe	Ser	
	405					410					415					

CAT His 420	CCC Pro	TCT Ser	CGG Arg	ATG Met	CCC Pro 425	GTC Val	TAC Tyr	CCC Pro	AAA Lys	TGG Trp 430	GTG Val	GGG Gly	GCC Ala	GAC Asp	CAT His 435	1455
GCA Ala	GAT Asp	GAC Asp	ATT Ile	CAG Gln 440	TAC Tyr	GTT Val	TTC Phe	GGG Gly	AAG Lys 445	CCC Pro	TTC Phe	GCC Ala	ACC Thr	CCC Pro 450	ACG Thr	1503
GGC Gly	TAC Tyr	CGG Arg	CCC Pro 455	CAA Gln	GAC Asp	AGG Arg	ACA Thr	GTC Val 460	TCT Ser	AAG Lys	GCC Ala	ATG Met	ATC Ile 465	GCC Ala	TAC Tyr	1551
TGG Trp	ACC Thr	AAC Asn 470	TTT Phe	GCC Ala	AAA Lys	ACA Thr	GGG Gly 475	GAC Asp	CCC Pro	AAC Asn	ATG Met	GGC Gly 480	GAC Asp	TCG Ser	GCT Ala	1599
GTG Val 485	CCC Pro	ACA Thr	CAC His	TGG Trp	GAA Glu 490	CCC Thr	TAC Tyr	ACT Thr	ACG Thr	GAA Glu 495	AAC Asn	AGC Ser	GGC Gly	TAC Tyr	CTG Leu	1647
GAG Glu 500	ATC Ile	ACC Thr	AAG Lys	AAG Lys	ATG Met 505	GGC Gly	AGC Ser	AGC Ser	TCC Ser	ATG Met 510	AAG Lys	CGG Arg	AGC Ser	CTG Leu	AGA Arg 515	1695
ACC Thr	AAC Asn	TTC Phe	CTG Leu	CGC Arg 520	TAC Tyr	TGG Trp	ACC Thr	CTC Leu	ACC Thr 525	TAT Tyr	CTG Leu	GCG Ala	CTG Leu	CCC Pro 530	ACA Thr	1743
GTG Val	ACC Thr	GAC Asp	CAG Gln 535	GAG Glu	GCC Ala	ACC Thr	CCT Pro	GTG Val 540	CCC Pro	CCC Pro	ACA Thr	GGG Gly	GAC Asp 545	TCC Ser	GAG Glu	1791
GCC Ala	ACT Thr	CCC Pro 550	GTG Val	CCC Pro	CCC Pro	ACG Thr	GGT Gly 555	GAC Asp	TCC Ser	GAG Glu	ACC Thr	GCC Ala 560	CCC Pro	GTG Val	CCG Pro	1839
CCC Pro	ACG Thr 565	GGT Gly	GAC Asp	TCC Ser	GGG Gly	GCC Ala 570	CCC Pro	CCC Pro	GTG Val	CCG Pro	CCC Pro 575	ACG Thr	GGT Gly	GAC Asp	TCC Ser	1887
GGG Gly 580	GCC Ala	CCC Pro	CCC Pro	GTG Val	CCG Pro 585	CCC Pro	ACG Thr	GGT Gly	GAC Asp	TCC Ser 590	GGG Gly	GCC Ala	CCC Pro	CCC Pro	GTG Val 595	1935
CCG Pro	CCC Pro	ACG Thr	GGT Gly	GAC Asp 600	TCC Ser	GGG Gly	GCC Ala	CCC Pro	CCC Pro 605	GTG Val	CCG Pro	CCC Pro	ACG Thr	GGT Gly 610	GAC Asp	1983
TCC Ser	GGG Gly	GCC Ala	CCC Pro 615	CCC Pro	GTG Val	CCG Pro	CCC Pro	ACG Thr 620	GGT Gly	GAC Asp	TCC Ser	GGG Gly	GCC Ala 625	CCC Pro	CCC Pro	2031
GTG Val	CCG Pro	CCC Pro 630	ACG Thr	GGT Gly	GAC Asp	TCC Ser	GGC Gly 635	GCC Ala	CCC Pro	CCC Pro	GTG Val	CCG Pro	CCC Pro 640	ACG Thr	GGT Gly	2079
GAC Asp	GCC Ala 645	GGG Gly	CCC Pro	CCC Pro	CCC Pro	GTG Val 650	CCG Pro	CCC Pro	ACG Thr	GGT Gly	GAC Asp 655	TCC Ser	GGC Gly	GCC Ala	CCC Pro	2127
CCC Pro 660	GTG Val	CCG Pro	CCC Pro	ACG Thr	GGT Gly 665	GAC Asp	TCC Ser	GGG Gly	GCC Ala	CCC Pro 670	CCC Pro	GTG Val	ACC Thr	CCC Pro	ACG Thr 675	2175
GGT Gly	GAC Asp	TCC Ser	GAG Glu	ACC Thr 680	GCC Ala	CCC Pro	GTG Val	CCG Pro	CCC Pro 685	ACG Thr	GGT Gly	GAC Asp	TCC Ser	GGG Gly 690	GCC Ala	2223

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CCC CCT GTG CCC CCC ACG GGT GAC TCT GAG GCT GCC CCT GTG CCC CCC      2271
Pro Pro Val Pro Pro Thr Gly Asp Ser Glu Ala Ala Pro Val Pro Pro
          695                      700                      705

ACA GAT GAC TCC AAG GAA GCT CAG ATG CCT GCA GTC ATT AGG TTT TAGCGTCCCA      2326
Thr Asp Asp Ser Lys Glu Ala Gln Met Pro Ala Val Ile Arg Phe
          710                      715                      720

TGAGCCTTGG TATCAAGAGG CCACAAGAGT GGGACCCAG GGGCTCCCCT CCCATCTTGA      2386

GCTCTTCCTG AATAAAGCCT CATACCCCTA AAAAAAAAAA AA                        2428

```

## (2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 745 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```

Met Leu Thr Met Gly Arg Leu Gln Leu Val Val Leu Gly Leu Thr Cys
-23          -20          -15          -10

Cys Trp Ala Val Ala Ser Ala Ala Lys Leu Gly Ala Val Tyr Thr Glu
      -5          1          5

Gly Gly Phe Val Glu Gly Val Asn Lys Lys Leu Gly Leu Leu Gly Asp
10      15      20      25

Ser Val Asp Ile Phe Lys Gly Ile Pro Phe Ala Ala Pro Thr Lys Ala
      30      35      40

Leu Glu Asn Pro Gln Pro His Pro Gly Trp Gln Gly Thr Leu Lys Ala
      45      50      55

Lys Asn Phe Lys Lys Arg Cys Leu Gln Ala Thr Ile Thr Gln Asp Ser
      60      65      70

Thr Tyr Gly Asp Glu Asp Cys Leu Tyr Leu Asn Ile Trp Val Pro Gln
      75      80      85

Gly Arg Lys Gln Val Ser Arg Asp Leu Pro Val Met Ile Trp Ile Tyr
90      95      100      105

Gly Gly Ala Phe Leu Met Gly Ser Gly His Gly Ala Asn Phe Leu Asn
      110      115      120

Asn Tyr Leu Tyr Asp Gly Glu Glu Ile Ala Thr Arg Gly Asn Val Ile
      125      130      135

Val Val Thr Phe Asn Tyr Arg Val Gly Pro Leu Gly Phe Leu Ser Thr
      140      145      150

Gly Asp Ala Asn Leu Pro Gly Asn Tyr Gly Leu Arg Asp Gln His Met
      155      160      165

Ala Ile Ala Trp Val Lys Arg Asn Ile Ala Ala Phe Gly Gly Asp Pro
170      175      180      185

Asn Asn Ile Thr Leu Phe Gly Glu Ser Ala Gly Gly Ala Ser Val Ser
      190      195      200

Leu Gln Thr Leu Ser Pro Tyr Asn Lys Gly Leu Ile Arg Arg Ala Ile
      205      210      215

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Ser Gln Ser Gly Val Ala Leu Ser Pro Trp Val Ile Gln Lys Asn Pro  
 220 225 230  
 Leu Phe Trp Ala Lys Lys Val Ala Glu Lys Val Gly Cys Pro Val Gly  
 235 240 245  
 Asp Ala Ala Arg Met Ala Gln Cys Leu Lys Val Thr Asp Pro Arg Ala  
 250 255 260 265  
 Leu Thr Leu Ala Tyr Lys Val Pro Leu Ala Gly Leu Glu Tyr Pro Met  
 270 275 280  
 Leu His Tyr Val Gly Phe Val Pro Val Ile Asp Gly Asp Phe Ile Pro  
 285 290 295  
 Ala Asp Pro Ile Asn Leu Tyr Ala Asn Ala Ala Asp Ile Asp Tyr Ile  
 300 305 310  
 Ala Gly Thr Asn Asn Met Asp Gly His Ile Phe Ala Ser Ile Asp Met  
 315 320 325  
 Pro Ala Ile Asn Lys Gly Asn Lys Lys Val Thr Glu Glu Asp Phe Tyr  
 330 335 340 345  
 Lys Leu Val Ser Glu Phe Thr Ile Thr Lys Gly Leu Arg Gly Ala Lys  
 350 355 360  
 Thr Thr Phe Asp Val Tyr Thr Glu Ser Trp Ala Gln Asp Pro Ser Gln  
 365 370 375  
 Glu Asn Lys Lys Lys Thr Val Val Asp Phe Glu Thr Asp Val Leu Phe  
 380 385 390  
 Leu Val Pro Thr Glu Ile Ala Leu Ala Gln His Arg Ala Asn Ala Lys  
 395 400 405  
 Ser Ala Lys Thr Tyr Ala Tyr Leu Phe Ser His Pro Ser Arg Met Pro  
 410 415 420 425  
 Val Tyr Pro Lys Trp Val Gly Ala Asp His Ala Asp Asp Ile Gln Tyr  
 430 435 440  
 Val Phe Gly Lys Pro Phe Ala Thr Pro Thr Gly Tyr Arg Pro Gln Asp  
 445 450 455  
 Arg Thr Val Ser Lys Ala Met Ile Ala Tyr Trp Thr Asn Phe Ala Lys  
 460 465 470  
 Thr Gly Asp Pro Asn Met Gly Asp Ser Ala Val Pro Thr His Trp Glu  
 475 480 485  
 Pro Tyr Thr Thr Glu Asn Ser Gly Tyr Leu Glu Ile Thr Lys Lys Met  
 490 495 500 505  
 Gly Ser Ser Ser Met Lys Arg Ser Leu Arg Thr Asn Phe Leu Arg Tyr  
 510 515 520  
 Trp Thr Leu Thr Tyr Leu Ala Leu Pro Thr Val Thr Asp Gln Glu Ala  
 525 530 535  
 Thr Pro Val Pro Pro Thr Gly Asp Ser Glu Ala Thr Pro Val Pro Pro  
 540 545 550  
 Thr Gly Asp Ser Glu Thr Ala Pro Val Pro Pro Thr Gly Asp Ser Gly  
 555 560 565  
 Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Gly Ala Pro Pro Val Pro  
 570 575 580 585

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 722 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(A) ORGANISM: Homo sapiens  
(F) TISSUE TYPE: Mammary gland

Ala 1	Lys	Leu	Gly	Ala 5	Val	Tyr	Thr	Glu	Gly 10	Gly	Phe	Val	Glu	Gly 15	Val
Asn	Lys	Lys	Leu 20	Gly	Leu	Leu	Gly	Asp 25	Ser	Val	Asp	Ile	Phe 30	Lys	Gly
Ile	Pro	Phe 35	Ala	Ala	Pro	Thr	Lys 40	Ala	Leu	Glu	Asn	Pro 45	Gln	Pro	His
Pro	Gly 50	Trp	Gln	Gly	Thr	Leu 55	Lys	Ala	Lys	Asn	Phe 60	Lys	Lys	Arg	Cys
Leu 65	Gln	Ala	Thr	Ile	Thr 70	Gln	Asp	Ser	Thr	Tyr 75	Gly	Asp	Glu	Asp	Cys 80
Leu	Tyr	Leu	Asn	Ile 85	Trp	Val	Pro	Gln	Gly 90	Arg	Lys	Gln	Val	Ser 95	Arg
Asp	Leu	Pro	Val 100	Met	Ile	Trp	Ile	Tyr 105	Gly	Gly	Ala	Phe	Leu 110	Met	Gly



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Ser	Gly	His	Gly	Ala	Asn	Phe	Leu	Asn	Asn	Tyr	Leu	Tyr	Asp	Gly	Glu		
		115					120					125					
Glu	Ile	Ala	Thr	Arg	Gly	Asn	Val	Ile	Val	Val	Thr	Phe	Asn	Tyr	Arg		
	130					135					140						
Val	Gly	Pro	Leu	Gly	Phe	Leu	Ser	Thr	Gly	Asp	Ala	Asn	Leu	Pro	Gly		
	145				150					155					160		
Asn	Tyr	Gly	Leu	Arg	Asp	Gln	His	Met	Ala	Ile	Ala	Trp	Val	Lys	Arg		
				165					170					175			
Asn	Ile	Ala	Ala	Phe	Gly	Gly	Asp	Pro	Asn	Asn	Ile	Thr	Leu	Phe	Gly		
			180					185					190				
Glu	Ser	Ala	Gly	Gly	Ala	Ser	Val	Ser	Leu	Gln	Thr	Leu	Ser	Pro	Tyr		
		195					200					205					
Asn	Lys	Gly	Leu	Ile	Arg	Arg	Ala	Ile	Ser	Gln	Ser	Gly	Val	Ala	Leu		
	210					215					220						
Ser	Pro	Trp	Val	Ile	Gln	Lys	Asn	Pro	Leu	Phe	Trp	Ala	Lys	Lys	Val		
	225				230					235					240		
Ala	Glu	Lys	Val	Gly	Cys	Pro	Val	Gly	Asp	Ala	Ala	Arg	Met	Ala	Gln		
				245					250					255			
Cys	Leu	Lys	Val	Thr	Asp	Pro	Arg	Ala	Leu	Thr	Leu	Ala	Tyr	Lys	Val		
			260					265					270				
Pro	Leu	Ala	Gly	Leu	Glu	Tyr	Pro	Met	Leu	His	Tyr	Val	Gly	Phe	Val		
		275					280					285					
Pro	Val	Ile	Asp	Gly	Asp	Phe	Ile	Pro	Ala	Asp	Pro	Ile	Asn	Leu	Tyr		
	290					295					300						
Ala	Asn	Ala	Ala	Asp	Ile	Asp	Tyr	Ile	Ala	Gly	Thr	Asn	Asn	Met	Asp		
	305				310					315				320			
Gly	His	Ile	Phe	Ala	Ser	Ile	Asp	Met	Pro	Ala	Ile	Asn	Lys	Gly	Asn		
				325					330					335			
Lys	Lys	Val	Thr	Glu	Glu	Asp	Phe	Tyr	Lys	Leu	Val	Ser	Glu	Phe	Thr		
			340					345					350				
Ile	Thr	Lys	Gly	Leu	Arg	Gly	Ala	Lys	Thr	Thr	Phe	Asp	Val	Tyr	Thr		
		355					360					365					
Glu	Ser	Trp	Ala	Gln	Asp	Pro	Ser	Gln	Glu	Asn	Lys	Lys	Lys	Thr	Val		
		370				375					380						
Val	Asp	Phe	Glu	Thr	Asp	Val	Leu	Phe	Leu	Val	Pro	Thr	Glu	Ile	Ala		
	385				390					395				400			
Leu	Ala	Gln	His	Arg	Ala	Asn	Ala	Lys	Ser	Ala	Lys	Thr	Tyr	Ala	Tyr		
				405					410					415			
Leu	Phe	Ser	His	Pro	Ser	Arg	Met	Pro	Val	Tyr	Pro	Lys	Trp	Val	Gly		
			420					425					430				
Ala	Asp	His	Ala	Asp	Asp	Ile	Gln	Tyr	Val	Phe	Gly	Lys	Pro	Phe	Ala		
		435					440					445					
Thr	Pro	Thr	Gly	Tyr	Arg	Pro	Gln	Asp	Arg	Thr	Val	Ser	Lys	Ala	Met		
	450					455					460						
Ile	Ala	Tyr	Trp	Thr	Asn	Phe	Ala	Lys	Thr	Gly	Asp	Pro	Asn	Met	Gly		
	465				470					475				480			

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Asp Ser Ala Val Pro Thr His Trp Glu Pro Tyr Thr Thr Glu Asn Ser  
 485 490 495  
 Gly Tyr Leu Glu Ile Thr Lys Lys Met Gly Ser Ser Ser Met Lys Arg  
 500 505 510  
 Ser Leu Arg Thr Asn Phe Leu Arg Tyr Trp Thr Leu Thr Tyr Leu Ala  
 515 520 525  
 Leu Pro Thr Val Thr Asp Gln Glu Ala Thr Pro Val Pro Pro Thr Gly  
 530 535 540  
 Asp Ser Glu Ala Thr Pro Val Pro Pro Thr Gly Asp Ser Glu Thr Ala  
 545 550 555 560  
 Pro Val Pro Pro Thr Gly Asp Ser Gly Ala Pro Pro Val Pro Pro Thr  
 565 570 575  
 Gly Asp Ser Gly Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Gly Ala  
 580 585 590  
 Pro Pro Val Pro Pro Thr Gly Asp Ser Gly Ala Pro Pro Val Pro Pro  
 595 600 605  
 Thr Gly Asp Ser Gly Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Gly  
 610 615 620  
 Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Gly Ala Pro Pro Val Pro  
 625 630 635 640  
 Pro Thr Gly Asp Ala Gly Pro Pro Pro Val Pro Pro Thr Gly Asp Ser  
 645 650 655  
 Gly Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Gly Ala Pro Pro Val  
 660 665 670  
 Thr Pro Thr Gly Asp Ser Glu Thr Ala Pro Val Pro Pro Thr Gly Asp  
 675 680 685  
 Ser Gly Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Glu Ala Ala Pro  
 690 695 700  
 Val Pro Pro Thr Asp Asp Ser Lys Glu Ala Gln Met Pro Ala Val Ile  
 705 710 715 720  
 Arg Phe

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 535 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (iii) HYPOTHETICAL: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (F) TISSUE TYPE: Mammary gland

## (ix) FEATURE:

- (A) NAME/KEY: Peptide
- (B) LOCATION: 1..535
- (D) OTHER INFORMATION: /label= Variant\_A

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala	Lys	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Gly	Phe	Val	Glu	Gly	Val	1	5	10	15
Asn	Lys	Lys	Leu	Gly	Leu	Leu	Gly	Asp	Ser	Val	Asp	Ile	Phe	Lys	Gly	20	25	30	
Ile	Pro	Phe	Ala	Ala	Pro	Thr	Lys	Ala	Leu	Glu	Asn	Pro	Gln	Pro	His	35	40	45	
Pro	Gly	Trp	Gln	Gly	Thr	Leu	Lys	Ala	Lys	Asn	Phe	Lys	Lys	Arg	Cys	50	55	60	
Leu	Gln	Ala	Thr	Ile	Thr	Gln	Asp	Ser	Thr	Tyr	Gly	Asp	Glu	Asp	Cys	65	70	75	80
Leu	Tyr	Leu	Asn	Ile	Trp	Val	Pro	Gln	Gly	Arg	Lys	Gln	Val	Ser	Arg	85	90	95	
Asp	Leu	Pro	Val	Met	Ile	Trp	Ile	Tyr	Gly	Gly	Ala	Phe	Leu	Met	Gly	100	105	110	
Ser	Gly	His	Gly	Ala	Asn	Phe	Leu	Asn	Asn	Tyr	Leu	Tyr	Asp	Gly	Glu	115	120	125	
Glu	Ile	Ala	Thr	Arg	Gly	Asn	Val	Ile	Val	Val	Thr	Phe	Asn	Tyr	Arg	130	135	140	
Val	Gly	Pro	Leu	Gly	Phe	Leu	Ser	Thr	Gly	Asp	Ala	Asn	Leu	Pro	Gly	145	150	155	160
Asn	Tyr	Gly	Leu	Arg	Asp	Gln	His	Met	Ala	Ile	Ala	Trp	Val	Lys	Arg	165	170	175	
Asn	Ile	Ala	Ala	Phe	Gly	Gly	Asp	Pro	Asn	Asn	Ile	Thr	Leu	Phe	Gly	180	185	190	
Glu	Ser	Ala	Gly	Gly	Ala	Ser	Val	Ser	Leu	Gln	Thr	Leu	Ser	Pro	Tyr	195	200	205	
Asn	Lys	Gly	Leu	Ile	Arg	Arg	Ala	Ile	Ser	Gln	Ser	Gly	Val	Ala	Leu	210	215	220	
Ser	Pro	Trp	Val	Ile	Gln	Lys	Asn	Pro	Leu	Phe	Trp	Ala	Lys	Lys	Val	225	230	235	240
Ala	Glu	Lys	Val	Gly	Cys	Pro	Val	Gly	Asp	Ala	Ala	Arg	Met	Ala	Gln	245	250	255	
Cys	Leu	Lys	Val	Thr	Asp	Pro	Arg	Ala	Leu	Thr	Leu	Ala	Tyr	Lys	Val	260	265	270	
Pro	Leu	Ala	Gly	Leu	Glu	Tyr	Pro	Met	Leu	His	Tyr	Val	Gly	Phe	Val	275	280	285	
Pro	Val	Ile	Asp	Gly	Asp	Phe	Ile	Pro	Ala	Asp	Pro	Ile	Asn	Leu	Tyr	290	295	300	
Ala	Asn	Ala	Ala	Asp	Ile	Asp	Tyr	Ile	Ala	Gly	Thr	Asn	Asn	Met	Asp	305	310	315	320
Gly	His	Ile	Phe	Ala	Ser	Ile	Asp	Met	Pro	Ala	Ile	Asn	Lys	Gly	Asn	325	330	335	
Lys	Lys	Val	Thr	Glu	Glu	Asp	Phe	Tyr	Lys	Leu	Val	Ser	Glu	Phe	Thr	340	345	350	

Ile	Thr	Lys 355	Gly	Leu	Arg	Gly	Ala 360	Lys	Thr	Thr	Phe	Asp 365	Val	Tyr	Thr
Glu	Ser	Trp	Ala	Gln	Asp	Pro 375	Ser	Gln	Glu	Asn	Lys 380	Lys	Lys	Thr	Val
Val 385	Asp	Phe	Glu	Thr	Asp 390	Val	Leu	Phe	Leu	Val 395	Pro	Thr	Glu	Ile	Ala 400
Leu	Ala	Gln	His	Arg 405	Ala	Asn	Ala	Lys	Ser 410	Ala	Lys	Thr	Tyr	Ala 415	Tyr
Leu	Phe	Ser	His 420	Pro	Ser	Arg	Met	Pro 425	Val	Tyr	Pro	Lys	Trp 430	Val	Gly
Ala	Asp	His 435	Ala	Asp	Asp	Ile	Gln 440	Tyr	Val	Phe	Gly	Lys 445	Pro	Phe	Ala
Thr	Pro 450	Thr	Gly	Tyr	Arg	Pro 455	Gln	Asp	Arg	Thr	Val 460	Ser	Lys	Ala	Met
Ile 465	Ala	Tyr	Trp	Thr	Asn 470	Phe	Ala	Lys	Thr	Gly 475	Asp	Pro	Asn	Met	Gly 480
Asp	Ser	Ala	Val	Pro 485	Thr	His	Trp	Glu	Pro 490	Tyr	Thr	Thr	Glu	Asn 495	Ser
Gly	Tyr	Leu	Glu 500	Ile	Thr	Lys	Lys	Met 505	Gly	Ser	Ser	Ser	Met 510	Lys	Arg
Ser	Leu	Arg 515	Thr	Asn	Phe	Leu	Arg 520	Tyr	Trp	Thr	Leu	Thr 525	Tyr	Leu	Ala
Leu	Pro 530	Thr	Val	Thr	Asp	Gln 535									

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 546 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens  
(F) TISSUE TYPE: Mammary gland

(ix) FEATURE:

- (A) NAME/KEY: Peptide  
(B) LOCATION: 1..546  
(D) OTHER INFORMATION: /label= Variant B

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ala	Lys	Leu	Gly	Ala	Val	Tyr	Thr	Glu	Gly	Gly	Phe	Val	Glu	Gly	Val
1				5					10					15	
Asn	Lys	Lys	Leu	Gly	Leu	Leu	Gly	Asp	Ser	Val	Asp	Ile	Phe	Lys	Gly
			20					25					30		
Ile	Pro	Phe	Ala	Ala	Pro	Thr	Lys	Ala	Leu	Glu	Asn	Pro	Gln	Pro	His
		35					40					45			

Pro 60	Gly 50	Trp	Gln	Gly	Thr	Leu 55	Lys	Ala	Lys	Asn	Phe 60	Lys	Lys	Arg	Cys
Leu 65	Gln	Ala	Thr	Ile	Thr 70	Gln	Asp	Ser	Thr	Tyr 75	Gly	Asp	Glu	Asp	Cys 80
Leu	Tyr	Leu	Asn	Ile 85	Trp	Val	Pro	Gln	Gly 90	Arg	Lys	Gln	Val	Ser 95	Arg
Asp	Leu	Pro	Val 100	Met	Ile	Trp	Ile	Tyr 105	Gly	Gly	Ala	Phe	Leu 110	Met	Gly
Ser	Gly	His 115	Gly	Ala	Asn	Phe	Leu 120	Asn	Asn	Tyr	Leu	Tyr 125	Asp	Gly	Glu
Glu	Ile 130	Ala	Thr	Arg	Gly	Asn 135	Val	Ile	Val	Val	Thr 140	Phe	Asn	Tyr	Arg
Val 145	Gly	Pro	Leu	Gly	Phe 150	Leu	Ser	Thr	Gly	Asp 155	Ala	Asn	Leu	Pro	Gly 160
Asn	Tyr	Gly	Leu	Arg 165	Asp	Gln	His	Met	Ala 170	Ile	Ala	Trp	Val	Lys 175	Arg
Asn	Ile	Ala	Ala 180	Phe	Gly	Gly	Asp	Pro 185	Asn	Asn	Ile	Thr	Leu 190	Phe	Gly
Glu	Ser	Ala 195	Gly	Gly	Ala	Ser	Val 200	Ser	Leu	Gln	Thr	Leu 205	Ser	Pro	Tyr
Asn	Lys 210	Gly	Leu	Ile	Arg	Arg 215	Ala	Ile	Ser	Gln	Ser 220	Gly	Val	Ala	Leu
Ser 225	Pro	Trp	Val	Ile	Gln 230	Lys	Asn	Pro	Leu	Phe 235	Trp	Ala	Lys	Lys	Val 240
Ala	Glu	Lys	Val	Gly 245	Cys	Pro	Val	Gly	Asp 250	Ala	Ala	Arg	Met	Ala 255	Gln
Cys	Leu	Lys	Val 260	Thr	Asp	Pro	Arg	Ala 265	Leu	Thr	Leu	Ala	Tyr 270	Lys	Val
Pro	Leu	Ala 275	Gly	Leu	Glu	Tyr	Pro 280	Met	Leu	His	Tyr	Val 285	Gly	Phe	Val
Pro	Val 290	Ile	Asp	Gly	Asp	Phe 295	Ile	Pro	Ala	Asp	Pro 300	Ile	Asn	Leu	Tyr
Ala 305	Asn	Ala	Ala	Asp	Ile 310	Asp	Tyr	Ile	Ala	Gly 315	Thr	Asn	Asn	Met	Asp 320
Gly	His	Ile	Phe	Ala 325	Ser	Ile	Asp	Met	Pro 330	Ala	Ile	Asn	Lys	Gly 335	Asn
Lys	Lys	Val	Thr 340	Glu	Glu	Asp	Phe	Tyr 345	Lys	Leu	Val	Ser	Glu 350	Phe	Thr
Ile	Thr	Lys 355	Gly	Leu	Arg	Gly	Ala 360	Lys	Thr	Thr	Phe	Asp 365	Val	Tyr	Thr
Glu	Ser 370	Trp	Ala	Gln	Asp	Pro 375	Ser	Gln	Glu	Asn	Lys 380	Lys	Lys	Thr	Val
Val 385	Asp	Phe	Glu	Thr	Asp 390	Val	Leu	Phe	Leu	Val 395	Pro	Thr	Glu	Ile	Ala 400
Leu	Ala	Gln	His	Arg 405	Ala	Asn	Ala	Lys	Ser 410	Ala	Lys	Thr	Tyr	Ala 415	Tyr

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Leu Phe Ser His Pro Ser Arg Met Pro Val Tyr Pro Lys Trp Val Gly
      420      425      430
Ala Asp His Ala Asp Asp Ile Gln Tyr Val Phe Gly Lys Pro Phe Ala
      435      440      445
Thr Pro Thr Gly Tyr Arg Pro Gln Asp Arg Thr Val Ser Lys Ala Met
      450      455      460
Ile Ala Tyr Trp Thr Asn Phe Ala Lys Thr Gly Asp Pro Asn Met Gly
      465      470      475      480
Asp Ser Ala Val Pro Thr His Trp Glu Pro Tyr Thr Thr Glu Asn Ser
      485      490      495
Gly Tyr Leu Glu Ile Thr Lys Lys Met Gly Ser Ser Ser Met Lys Arg
      500      505      510
Ser Leu Arg Thr Asn Phe Leu Arg Tyr Trp Thr Leu Thr Tyr Leu Ala
      515      520      525
Leu Pro Thr Val Thr Asp Gln Lys Glu Ala Gln Met Pro Ala Val Ile
      530      535      540
Arg Phe
545

```

## (2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 568 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
  - (F) TISSUE TYPE: Mammary gland
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION: 1..568
  - (D) OTHER INFORMATION: /label= Variant\_C

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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Ala Lys Leu Gly Ala Val Tyr Thr Glu Gly Gly Phe Val Glu Gly Val
1      5      10      15
Asn Lys Lys Leu Gly Leu Leu Gly Asp Ser Val Asp Ile Phe Lys Gly
      20      25      30
Ile Pro Phe Ala Ala Pro Thr Lys Ala Leu Glu Asn Pro Gln Pro His
      35      40      45
Pro Gly Trp Gln Gly Thr Leu Lys Ala Lys Asn Phe Lys Lys Arg Cys
      50      55      60
Leu Gln Ala Thr Ile Thr Gln Asp Ser Thr Tyr Gly Asp Glu Asp Cys
      65      70      75      80
Leu Tyr Leu Asn Ile Trp Val Pro Gln Gly Arg Lys Gln Val Ser Arg
      85      90      95

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Asp	Leu	Pro	Val	Met	Ile	Trp	Ile	Tyr	Gly	Gly	Ala	Phe	Leu	Met	Gly	100	105	110
Ser	Gly	His	Gly	Ala	Asn	Phe	Leu	Asn	Asn	Tyr	Leu	Tyr	Asp	Gly	Glu	115	120	125
Glu	Ile	Ala	Thr	Arg	Gly	Asn	Val	Ile	Val	Val	Thr	Phe	Asn	Tyr	Arg	130	135	140
Val	Gly	Pro	Leu	Gly	Phe	Leu	Ser	Thr	Gly	Asp	Ala	Asn	Leu	Pro	Gly	145	150	155
Asn	Tyr	Gly	Leu	Arg	Asp	Gln	His	Met	Ala	Ile	Ala	Trp	Val	Lys	Arg	165	170	175
Asn	Ile	Ala	Ala	Phe	Gly	Gly	Asp	Pro	Asn	Asn	Ile	Thr	Leu	Phe	Gly	180	185	190
Glu	Ser	Ala	Gly	Gly	Ala	Ser	Val	Ser	Leu	Gln	Thr	Leu	Ser	Pro	Tyr	195	200	205
Asn	Lys	Gly	Leu	Ile	Arg	Arg	Ala	Ile	Ser	Gln	Ser	Gly	Val	Ala	Leu	210	215	220
Ser	Pro	Trp	Val	Ile	Gln	Lys	Asn	Pro	Leu	Phe	Trp	Ala	Lys	Lys	Val	225	230	235
Ala	Glu	Lys	Val	Gly	Cys	Pro	Val	Gly	Asp	Ala	Ala	Arg	Met	Ala	Gln	245	250	255
Cys	Leu	Lys	Val	Thr	Asp	Pro	Arg	Ala	Leu	Thr	Leu	Ala	Tyr	Lys	Val	260	265	270
Pro	Leu	Ala	Gly	Leu	Glu	Tyr	Pro	Met	Leu	His	Tyr	Val	Gly	Phe	Val	275	280	285
Pro	Val	Ile	Asp	Gly	Asp	Phe	Ile	Pro	Ala	Asp	Pro	Ile	Asn	Leu	Tyr	290	295	300
Ala	Asn	Ala	Ala	Asp	Ile	Asp	Tyr	Ile	Ala	Gly	Thr	Asn	Asn	Met	Asp	305	310	315
Gly	His	Ile	Phe	Ala	Ser	Ile	Asp	Met	Pro	Ala	Ile	Asn	Lys	Gly	Asn	325	330	335
Lys	Lys	Val	Thr	Glu	Glu	Asp	Phe	Tyr	Lys	Leu	Val	Ser	Glu	Phe	Thr	340	345	350
Ile	Thr	Lys	Gly	Leu	Arg	Gly	Ala	Lys	Thr	Thr	Phe	Asp	Val	Tyr	Thr	355	360	365
Glu	Ser	Trp	Ala	Gln	Asp	Pro	Ser	Gln	Glu	Asn	Lys	Lys	Lys	Thr	Val	370	375	380
Val	Asp	Phe	Glu	Thr	Asp	Val	Leu	Phe	Leu	Val	Pro	Thr	Glu	Ile	Ala	385	390	395
Leu	Ala	Gln	His	Arg	Ala	Asn	Ala	Lys	Ser	Ala	Lys	Thr	Tyr	Ala	Tyr	405	410	415
Leu	Phe	Ser	His	Pro	Ser	Arg	Met	Pro	Val	Tyr	Pro	Lys	Trp	Val	Gly	420	425	430
Ala	Asp	His	Ala	Asp	Asp	Ile	Gln	Tyr	Val	Phe	Gly	Lys	Pro	Phe	Ala	435	440	445
Thr	Pro	Thr	Gly	Tyr	Arg	Pro	Gln	Asp	Arg	Thr	Val	Ser	Lys	Ala	Met	450	455	460

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Ile Ala Tyr Trp Thr Asn Phe Ala Lys Thr Gly Asp Pro Asn Met Gly  
 465 470 475 480  
 Asp Ser Ala Val Pro Thr His Trp Glu Pro Tyr Thr Thr Glu Asn Ser  
 485 490 495  
 Gly Tyr Leu Glu Ile Thr Lys Lys Met Gly Ser Ser Ser Met Lys Arg  
 500 505 510  
 Ser Leu Arg Thr Asn Phe Leu Arg Tyr Trp Thr Leu Thr Tyr Leu Ala  
 515 520 525  
 Leu Pro Thr Val Thr Asp Gln Gly Ala Pro Pro Val Pro Pro Thr Gly  
 530 535 540  
 Asp Ser Gly Ala Pro Pro Val Pro Pro Thr Gly Asp Ser Lys Glu Ala  
 545 550 555 560  
 Gln Met Pro Ala Val Ile Arg Phe  
 565

## (2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 722 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
  - (F) TISSUE TYPE: Mammary gland
- (ix) FEATURE:
  - (A) NAME/KEY: Peptide
  - (B) LOCATION: 1..722
  - (D) OTHER INFORMATION: /label= Variant\_N

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ala Lys Leu Gly Ala Val Tyr Thr Glu Gly Gly Phe Val Glu Gly Val  
 1 5 10 15  
 Asn Lys Lys Leu Gly Leu Leu Gly Asp Ser Val Asp Ile Phe Lys Gly  
 20 25 30  
 Ile Pro Phe Ala Ala Pro Thr Lys Ala Leu Glu Asn Pro Gln Pro His  
 35 40 45  
 Pro Gly Trp Gln Gly Thr Leu Lys Ala Lys Asn Phe Lys Lys Arg Cys  
 50 55 60  
 Leu Gln Ala Thr Ile Thr Gln Asp Ser Thr Tyr Gly Asp Glu Asp Cys  
 65 70 75 80  
 Leu Tyr Leu Asn Ile Trp Val Pro Gln Gly Arg Lys Gln Val Ser Arg  
 85 90 95  
 Asp Leu Pro Val Met Ile Trp Ile Tyr Gly Gly Ala Phe Leu Met Gly  
 100 105 110  
 Ser Gly His Gly Ala Asn Phe Leu Asn Asn Tyr Leu Tyr Asp Gly Glu  
 115 120 125



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Glu	Ile	Ala	Thr	Arg	Gly	Asn	Val	Ile	Val	Val	Thr	Phe	Asn	Tyr	Arg		
130						135					140						
Val	Gly	Pro	Leu	Gly	Phe	Leu	Ser	Thr	Gly	Asp	Ala	Asn	Leu	Pro	Gly		
145					150					155					160		
Asn	Tyr	Gly	Leu	Arg	Asp	Gln	His	Met	Ala	Ile	Ala	Trp	Val	Lys	Arg		
				165					170					175			
Asn	Ile	Ala	Ala	Phe	Gly	Gly	Asp	Pro	Asn	Gln	Ile	Thr	Leu	Phe	Gly		
			180					185					190				
Glu	Ser	Ala	Gly	Gly	Ala	Ser	Val	Ser	Leu	Gln	Thr	Leu	Ser	Pro	Tyr		
		195					200					205					
Asn	Lys	Gly	Leu	Ile	Arg	Arg	Ala	Ile	Ser	Gln	Ser	Gly	Val	Ala	Leu		
210						215					220						
Ser	Pro	Trp	Val	Ile	Gln	Lys	Asn	Pro	Leu	Phe	Trp	Ala	Lys	Lys	Val		
225					230					235							
Ala	Glu	Lys	Val	Gly	Cys	Pro	Val	Gly	Asp	Ala	Ala	Arg	Met	Ala	Gln		
				245					250					255			
Cys	Leu	Lys	Val	Thr	Asp	Pro	Arg	Ala	Leu	Thr	Leu	Ala	Tyr	Lys	Val		
			260					265					270				
Pro	Leu	Ala	Gly	Leu	Glu	Tyr	Pro	Met	Leu	His	Tyr	Val	Gly	Phe	Val		
		275					280					285					
Pro	Val	Ile	Asp	Gly	Asp	Phe	Ile	Pro	Ala	Asp	Pro	Ile	Asn	Leu	Tyr		
	290					295					300						
Ala	Asn	Ala	Ala	Asp	Ile	Asp	Tyr	Ile	Ala	Gly	Thr	Asn	Asn	Met	Asp		
305					310					315					320		
Gly	His	Ile	Phe	Ala	Ser	Ile	Asp	Met	Pro	Ala	Ile	Asn	Lys	Gly	Asn		
				325					330					335			
Lys	Lys	Val	Thr	Glu	Glu	Asp	Phe	Tyr	Lys	Leu	Val	Ser	Glu	Phe	Thr		
			340					345					350				
Ile	Thr	Lys	Gly	Leu	Arg	Gly	Ala	Lys	Thr	Thr	Phe	Asp	Val	Tyr	Thr		
		355					360					365					
Glu	Ser	Trp	Ala	Gln	Asp	Pro	Ser	Gln	Glu	Asn	Lys	Lys	Lys	Thr	Val		
	370					375					380						
Val	Asp	Phe	Glu	Thr	Asp	Val	Leu	Phe	Leu	Val	Pro	Thr	Glu	Ile	Ala		
385					390					395					400		
Leu	Ala	Gln	His	Arg	Ala	Asn	Ala	Lys	Ser	Ala	Lys	Thr	Tyr	Ala	Tyr		
				405					410					415			
Leu	Phe	Ser	His	Pro	Ser	Arg	Met	Pro	Val	Tyr	Pro	Lys	Trp	Val	Gly		
			420					425					430				
Ala	Asp	His	Ala	Asp	Asp	Ile	Gln	Tyr	Val	Phe	Gly	Lys	Pro	Phe	Ala		
		435					440					445					
Thr	Pro	Thr	Gly	Tyr	Arg	Pro	Gln	Asp	Arg	Thr	Val	Ser	Lys	Ala	Met		
	450					455					460						
Ile	Ala	Tyr	Trp	Thr	Asn	Phe	Ala	Lys	Thr	Gly	Asp	Pro	Asn	Met	Gly		
465					470					475					480		
Asp	Ser	Ala	Val	Pro	Thr	His	Trp	Glu	Pro	Tyr	Thr	Thr	Glu	Asn	Ser		
				485					490					495			

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Gly	Tyr	Leu	Glu	Ile	Thr	Lys	Lys	Met	Gly	Ser	Ser	Ser	Met	Lys	Arg
			500					505					510		
Ser	Leu	Arg	Thr	Asn	Phe	Leu	Arg	Tyr	Trp	Thr	Leu	Thr	Tyr	Leu	Ala
		515					520					525			
Leu	Pro	Thr	Val	Thr	Asp	Gln	Glu	Ala	Thr	Pro	Val	Pro	Pro	Thr	Gly
	530					535					540				
Asp	Ser	Glu	Ala	Thr	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Glu	Thr	Ala
545					550					555					560
Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr
				565					570						575
Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala
			580					585					590		
Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro
		595					600						605		
Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly
	610					615						620			
Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro
625						630				635					640
Pro	Thr	Gly	Asp	Ala	Gly	Pro	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser
				645					650					655	
Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val
			660					665					670		
Thr	Pro	Thr	Gly	Asp	Ser	Glu	Thr	Ala	Pro	Val	Pro	Pro	Thr	Gly	Asp
		675					680						685		
Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Glu	Ala	Ala	Pro
	690					695					700				
Val	Pro	Pro	Thr	Asp	Asp	Ser	Lys	Glu	Ala	Gln	Met	Pro	Ala	Val	Ile
705					710					715					720

Arg Phe

## (2) INFORMATION FOR SEQ ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2184 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iii) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens
- (F) TISSUE TYPE: mammary gland

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 82..2088
- (D) OTHER INFORMATION: /label= Variant\_T

- (ix) FEATURE:  
 (A) NAME/KEY: mat\_peptide  
 (B) LOCATION: 151..2085
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_region  
 (B) LOCATION: 1756..2052
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1756..1788
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1789..1821
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1822..1854
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1855..1887
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1888..1920
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1921..1953
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1954..1986
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 1987..2019
- (ix) FEATURE:  
 (A) NAME/KEY: repeat\_unit  
 (B) LOCATION: 2020..2052

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACCTTCTGTA TCAGTTAAGT GTCAAGATGG AAGGAACAGC AGTCTCAAGA TAATGCAAAG	60
AGTTTATTCA TCCAGAGGCT G ATG CTC ACC ATG GGG CGC CTG CAA CTG GTT	111
Met Leu Thr Met Gly Arg Leu Gln Leu Val	
-23 -20 -15	
GTG TTG GGC CTC ACC TGC TGC TGG GCA GTG GCG AGT GCC GCG AAG CTG	159
Val Leu Gly Leu Thr Cys Cys Trp Ala Val Ala Ser Ala Ala Lys Leu	
-10 -5 1	
GGC GCC GTG TAC ACA GAA GGT GGG TTC GTG GAA GGC GTC AAT AAG AAG	207
Gly Ala Val Tyr Thr Glu Gly Gly Phe Val Glu Gly Val Asn Lys Lys	
5 10 15	
CTC GGC CTC CTG GGT GAC TCT GTG GAC ATC TTC AAG GGC ATC CCC TTC	255
Leu Gly Leu Leu Gly Asp Ser Val Asp Ile Phe Lys Gly Ile Pro Phe	
20 25 30 35	

GCA	GCT	CCC	ACC	AAG	GCC	CTG	GAA	AAT	CCT	CAG	CCA	CAT	CCT	GGC	TGG	303
Ala	Ala	Pro	Thr	Lys	Ala	Leu	Glu	Asn	Pro	Gln	Pro	His	Pro	Gly	Trp	
				40					45					50		
CAA	GGG	ACC	CTG	AAG	GCC	AAG	AAC	TTC	AAG	AAG	AGA	TGC	CTG	CAG	GCC	351
Gln	Gly	Thr	Leu	Lys	Ala	Lys	Asn	Phe	Lys	Lys	Arg	Cys	Leu	Gln	Ala	
			55					60					65			
ACC	ATC	ACC	CAG	GAC	AGC	ACC	TAC	GGG	GAT	GAA	GAC	TGC	CTG	TAC	CTC	399
Thr	Ile	Thr	Gln	Asp	Ser	Thr	Tyr	Gly	Asp	Glu	Asp	Cys	Leu	Tyr	Leu	
		70					75					80				
AAC	ATT	TGG	GTG	CCC	CAG	GGC	AGG	AAG	CAA	GTC	TCC	CGG	GAC	CTG	CCC	447
Asn	Ile	Trp	Val	Pro	Gln	Gly	Arg	Lys	Gln	Val	Ser	Arg	Asp	Leu	Pro	
	85					90					95					
GTT	ATG	ATC	TGG	ATC	TAT	GGA	GGC	GCC	TTC	CTC	ATG	GGG	TCC	GGC	CAT	495
Val	Met	Ile	Trp	Ile	Tyr	Gly	Gly	Ala	Phe	Leu	Met	Gly	Ser	Gly	His	
100					105					110					115	
GGG	GCC	AAC	TTC	CTC	AAC	AAC	TAC	CTG	TAT	GAC	GGC	GAG	GAG	ATC	GCC	543
Gly	Ala	Asn	Phe	Leu	Asn	Asn	Tyr	Leu	Tyr	Asp	Gly	Glu	Glu	Ile	Ala	
				120					125					130		
ACA	CGC	GGA	AAC	GTC	ATC	GTG	GTC	ACC	TTC	AAC	TAC	CGT	GTC	GGC	CCC	591
Thr	Arg	Gly	Asn	Val	Ile	Val	Val	Thr	Phe	Asn	Tyr	Arg	Val	Gly	Pro	
			135					140					145			
CTT	GGG	TTC	CTC	AGC	ACT	GGG	GAC	GCC	AAT	CTG	CCA	GGT	AAC	TAT	GGC	639
Leu	Gly	Phe	Leu	Ser	Thr	Gly	Asp	Ala	Asn	Leu	Pro	Gly	Asn	Tyr	Gly	
		150					155					160				
CTT	CGG	GAT	CAG	CAC	ATG	GCC	ATT	GCT	TGG	GTG	AAG	AGG	AAT	ATC	GCG	687
Leu	Arg	Asp	Gln	His	Met	Ala	Ile	Ala	Trp	Val	Lys	Arg	Asn	Ile	Ala	
	165					170					175					
GCC	TTC	GGG	GGG	GAC	CCC	AAC	AAC	ATC	ACG	CTC	TTC	GGG	GAG	TCT	GCT	735
Ala	Phe	Gly	Gly	Asp	Pro	Asn	Asn	Ile	Thr	Leu	Phe	Gly	Glu	Ser	Ala	
180					185					190					195	
GGA	GGT	GCC	AGC	GTC	TCT	CTG	CAG	ACC	CTC	TCC	CCC	TAC	AAC	AAG	GGC	783
Gly	Gly	Ala	Ser	Val	Ser	Leu	Gln	Thr	Leu	Ser	Pro	Tyr	Asn	Lys	Gly	
				200					205					210		
CTC	ATC	CGG	CGA	GCC	ATC	AGC	CAG	AGC	GGC	GTG	GCC	CTG	AGT	CCC	TGG	831
Leu	Ile	Arg	Arg	Ala	Ile	Ser	Gln	Ser	Gly	Val	Ala	Leu	Ser	Pro	Trp	
			215					220					225			
GTC	ATC	CAG	AAA	AAC	CCA	CTC	TTC	TGG	GCC	AAA	AAG	GTG	GCT	GAG	AAG	879
Val	Ile	Gln	Lys	Asn	Pro	Leu	Phe	Trp	Ala	Lys	Lys	Val	Ala	Glu	Lys	
		230					235					240				
GTG	GGT	TGC	CCT	GTG	GGT	GAT	GCC	GCC	AGG	ATG	GCC	CAG	TGT	CTG	AAG	927
Val	Gly	Cys	Pro	Val	Gly	Asp	Ala	Ala	Arg	Met	Ala	Gln	Cys	Leu	Lys	
	245					250					255					
GTT	ACT	GAT	CCC	CGA	GCC	CTG	ACG	CTG	GCC	TAT	AAG	GTG	CCG	CTG	GCA	975
Val	Thr	Asp	Pro	Arg	Ala	Leu	Thr	Leu	Ala	Tyr	Lys	Val	Pro	Leu	Ala	
260					265					270					275	
GGC	CTG	GAG	TAC	CCC	ATG	CTG	CAC	TAT	GTG	GGC	TTC	GTC	CCT	GTC	ATT	1023
Gly	Leu	Glu	Tyr	Pro	Met	Leu	His	Tyr	Val	Gly	Phe	Val	Pro	Val	Ile	
				280					285					290		
GAT	GGA	GAC	TTC	ATC	CCC	GCT	GAC	CCG	ATC	AAC	CTG	TAC	GCC	AAC	GCC	1071
Asp	Gly	Asp	Phe	Ile	Pro	Ala	Asp	Pro	Ile	Asn	Leu	Tyr	Ala	Asn	Ala	
			295					300					305			

GCC Ala	GAC Asp	ATC Ile 310	GAC Asp	TAT Tyr	ATA Ile	GCA Ala	GGC Gly 315	ACC Thr	AAC Asn	AAC Asn	ATG Met	GAC Asp 320	GGC Gly	CAC His	ATC Ile	1119
TTC Phe	GCC Ala 325	AGC Ser	ATC Ile	GAC Asp	ATG Met	CCT Pro 330	GGC Ala	ATC Ile	AAC Asn	AAG Lys	GGC Gly 335	AAC Asn	AAG Lys	AAA Lys	GTC Val	1167
ACG Thr 340	GAG Glu	GAG Glu	GAC Asp	TTC Phe	TAC Tyr 345	AAG Lys	CTG Leu	GTC Val	AGT Ser	GAG Glu 350	TTC Phe	ACA Thr	ATC Ile	ACC Thr	AAG Lys 355	1215
GGG Gly	CTC Leu	AGA Arg	GGC Gly	GCC Ala 360	AAG Lys	ACG Thr	ACC Thr	TTT Phe	GAT Asp 365	GTC Val	TAC Tyr	ACC Thr	GAG Glu	TCC Ser 370	TGG Trp	1263
GCC Ala	CAG Gln	GAC Asp 375	CCA Pro	TCC Ser	CAG Gln	GAG Glu	AAT Asn 380	AAG Lys	AAG Lys	AAG Lys	ACT Thr	GTG Val	GTG Val 385	GAC Asp	TTT Phe	1311
GAG Glu	ACC Thr	GAT Asp 390	GTC Val	CTC Leu	TTC Phe	CTG Leu 395	GTG Val	CCC Pro	ACC Thr	GAG Glu	ATT Ile	GCC Ala 400	CTA Leu	GCC Ala	CAG Gln	1359
CAC His 405	AGA Arg	GCC Ala	AAT Asn	GCC Ala	AAG Lys	AGT Ser 410	GCC Ala	AAG Lys	ACC Thr	TAC Tyr	GCC Ala 415	TAC Tyr	CTG Leu	TTT Phe	TCC Ser	1407
CAT His 420	CCC Pro	TCT Ser	CGG Arg	ATG Met	CCC Pro 425	GTC Val	TAC Tyr	CCC Pro	AAA Lys	TGG Trp 430	GTG Val	GGG Gly	GCC Ala	GAC Asp	CAT His 435	1455
GCA Ala	GAT Asp	GAC Asp	ATT Ile 440	CAG Gln	TAC Tyr	GTT Val	TTC Phe	GGG Gly 445	AAG Lys	CCC Pro	TTC Phe	GCC Ala	ACC Thr	CCC Pro 450	ACG Thr	1503
GGC Gly	TAC Tyr	CGG Arg	CCC Pro 455	CAA Gln	GAC Asp	AGG Arg	ACA Thr	GTC Val 460	TCT Ser	AAG Lys	GCC Ala	ATG Met	ATC Ile 465	GCC Ala	TAC Tyr	1551
TGG Trp	ACC Thr	AAC Asn 470	TTT Phe	GCC Ala	AAA Lys	ACA Thr	GGG Gly 475	GAC Asp	CCC Pro	AAC Asn	ATG Met	GGC Gly 480	GAC Asp	TCG Ser	GCT Ala	1599
GTG Val 485	CCC Pro	ACA Thr	CAC His	TGG Trp	GAA Glu	CCC Pro 490	TAC Tyr	ACT Thr	ACG Thr	GAA Glu	AAC Asn 495	AGC Ser	GGC Gly	TAC Tyr	CTG Leu	1647
GAG Glu 500	ATC Ile	ACC Thr	AAG Lys	AAG Lys	ATG Met 505	GGC Gly	AGC Ser	AGC Ser	TCC Ser	ATG Met 510	AAG Lys	CGG Arg	AGC Ser	CTG Leu	AGA Arg 515	1695
ACC Thr	AAC Asn	TTC Phe	CTG Leu	CGC Arg 520	TAC Tyr	TGG Trp	ACC Thr	CTC Leu	ACC Thr 525	TAT Tyr	CTG Leu	GCG Ala	CTG Leu	CCC Pro 530	ACA Thr	1743
GTG Val	ACC Thr	GAC Asp	CAG Gln 535	GAG Glu	GCC Ala	ACC Thr	CCT Pro	GTG Val 540	CCC Pro	CCC Pro	ACA Thr	GGG Gly	GAC Asp 545	TCC Ser	GAG Glu	1791
GCC Ala	ACT Thr	CCC Pro 550	GTG Val	CCC Pro	CCC Pro	ACG Thr	GGT Gly 555	GAC Asp	TCC Ser	GAG Glu	ACC Thr	GCC Ala 560	CCC Pro	GTG Val	CCG Pro	1839
CCC Pro	ACG Thr 565	GGT Gly	GAC Asp	TCC Ser	GGG Gly	GCC Ala 570	CCC Pro	CCC Pro	GTG Val	CCG Pro	CCC Pro 575	ACG Thr	GGT Gly	GAC Asp	TCC Ser	1887

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GGG	GCC	CCC	CCC	GTG	CCG	CCC	ACG	GGT	GAC	TCC	GGG	GCC	CCC	CCC	GTG	1935
Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	
580					585					590					595	
CCG	CCC	ACG	GGT	GAC	TCC	GGG	GCC	CCC	CCC	GTG	CCG	CCC	ACG	GGT	GAC	1983
Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	
				600						605					610	
TCC	GGG	GCC	CCC	CCC	GTG	CCG	CCC	ACG	GGT	GAC	TCC	GGG	GCC	CCC	CCT	2031
Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	
			615					620					625			
GTG	CCC	CCC	ACA	GAT	GAC	TCC	AAG	GAA	GCT	CAG	ATG	CCT	GCA	GTC	ATT	2079
Val	Pro	Pro	Thr	Asp	Asp	Ser	Lys	Glu	Ala	Gln	Met	Pro	Ala	Val	Ile	
		630					635					640				
AGG	TTT	TAGCGTCCCA TGAGCCTTGG TATCAAGAGG CCACAAGAGT GGGACCCCAG										2135				
Arg	Phe															
	645															
GGGCTCCCCT	CCCATCTTGA	GCTCTTCCTG	AATAAAGCCT	CATACCCCT												2184

## (2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 668 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Met	Leu	Thr	Met	Gly	Arg	Leu	Gln	Leu	Val	Val	Leu	Gly	Leu	Thr	Cys
-23			-20				-15						-10		
Cys	Trp	Ala	Val	Ala	Ser	Ala	Ala	Lys	Leu	Gly	Ala	Val	Tyr	Thr	Glu
	-5						1				5				
Gly	Gly	Phe	Val	Glu	Gly	Val	Asn	Lys	Lys	Leu	Gly	Leu	Leu	Gly	Asp
10				15						20					25
Ser	Val	Asp	Ile	Phe	Lys	Gly	Ile	Pro	Phe	Ala	Ala	Pro	Thr	Lys	Ala
			30					35						40	
Leu	Glu	Asn	Pro	Gln	Pro	His	Pro	Gly	Trp	Gln	Gly	Thr	Leu	Lys	Ala
		45					50						55		
Lys	Asn	Phe	Lys	Lys	Arg	Cys	Leu	Gln	Ala	Thr	Ile	Thr	Gln	Asp	Ser
	60					65					70				
Thr	Tyr	Gly	Asp	Glu	Asp	Cys	Leu	Tyr	Leu	Asn	Ile	Trp	Val	Pro	Gln
	75					80				85					
Gly	Arg	Lys	Gln	Val	Ser	Arg	Asp	Leu	Pro	Val	Met	Ile	Trp	Ile	Tyr
90					95					100					105
Gly	Gly	Ala	Phe	Leu	Met	Gly	Ser	Gly	His	Gly	Ala	Asn	Phe	Leu	Asn
			110						115					120	
Asn	Tyr	Leu	Tyr	Asp	Gly	Glu	Glu	Ile	Ala	Thr	Arg	Gly	Asn	Val	Ile
		125						130					135		
Val	Val	Thr	Phe	Asn	Tyr	Arg	Val	Gly	Pro	Leu	Gly	Phe	Leu	Ser	Thr
		140					145					150			

Gly Asp Ala Asn Leu Pro Gly Asn Tyr Gly Leu Arg Asp Gln His Met  
 155 160 165  
 Ala Ile Ala Trp Val Lys Arg Asn Ile Ala Ala Phe Gly Gly Asp Pro  
 170 175 180 185  
 Asn Asn Ile Thr Leu Phe Gly Glu Ser Ala Gly Gly Ala Ser Val Ser  
 190 195 200  
 Leu Gln Thr Leu Ser Pro Tyr Asn Lys Gly Leu Ile Arg Arg Ala Ile  
 205 210 215  
 Ser Gln Ser Gly Val Ala Leu Ser Pro Trp Val Ile Gln Lys Asn Pro  
 220 225 230  
 Leu Phe Trp Ala Lys Lys Val Ala Glu Lys Val Gly Cys Pro Val Gly  
 235 240 245  
 Asp Ala Ala Arg Met Ala Gln Cys Leu Lys Val Thr Asp Pro Arg Ala  
 250 255 260 265  
 Leu Thr Leu Ala Tyr Lys Val Pro Leu Ala Gly Leu Glu Tyr Pro Met  
 270 275 280  
 Leu His Tyr Val Gly Phe Val Pro Val Ile Asp Gly Asp Phe Ile Pro  
 285 290 295  
 Ala Asp Pro Ile Asn Leu Tyr Ala Asn Ala Ala Asp Ile Asp Tyr Ile  
 300 305 310  
 Ala Gly Thr Asn Asn Met Asp Gly His Ile Phe Ala Ser Ile Asp Met  
 315 320 325  
 Pro Ala Ile Asn Lys Gly Asn Lys Lys Val Thr Glu Glu Asp Phe Tyr  
 330 335 340 345  
 Lys Leu Val Ser Glu Phe Thr Ile Thr Lys Gly Leu Arg Gly Ala Lys  
 350 355 360  
 Thr Thr Phe Asp Val Tyr Thr Glu Ser Trp Ala Gln Asp Pro Ser Gln  
 365 370 375  
 Glu Asn Lys Lys Lys Thr Val Val Asp Phe Glu Thr Asp Val Leu Phe  
 380 385 390  
 Leu Val Pro Thr Glu Ile Ala Leu Ala Gln His Arg Ala Asn Ala Lys  
 395 400 405  
 Ser Ala Lys Thr Tyr Ala Tyr Leu Phe Ser His Pro Ser Arg Met Pro  
 410 415 420 425  
 Val Tyr Pro Lys Trp Val Gly Ala Asp His Ala Asp Asp Ile Gln Tyr  
 430 435 440  
 Val Phe Gly Lys Pro Phe Ala Thr Pro Thr Gly Tyr Arg Pro Gln Asp  
 445 450 455  
 Arg Thr Val Ser Lys Ala Met Ile Ala Tyr Trp Thr Asn Phe Ala Lys  
 460 465 470  
 Thr Gly Asp Pro Asn Met Gly Asp Ser Ala Val Pro Thr His Trp Glu  
 475 480 485  
 Pro Tyr Thr Thr Glu Asn Ser Gly Tyr Leu Glu Ile Thr Lys Lys Met  
 490 495 500 505  
 Gly Ser Ser Ser Met Lys Arg Ser Leu Arg Thr Asn Phe Leu Arg Tyr  
 510 515 520

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Trp	Thr	Leu	Thr	Tyr	Leu	Ala	Leu	Pro	Thr	Val	Thr	Asp	Gln	Glu	Ala
			525					530					535		
Thr	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Glu	Ala	Thr	Pro	Val	Pro	Pro
		540					545					550			
Thr	Gly	Asp	Ser	Glu	Thr	Ala	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly
	555					560					565				
Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro
570					575					580					585
Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser
				590					595					600	
Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val
			605					610					615		
Pro	Pro	Thr	Gly	Asp	Ser	Gly	Ala	Pro	Pro	Val	Pro	Pro	Thr	Asp	Asp
		620					625					630			
Ser	Lys	Glu	Ala	Gln	Met	Pro	Ala	Val	Ile	Arg	Phe				
	635					640					645				



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Applicant's or agent's filing reference number	HX 1185-1 WO	International application No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSMZ)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 12 June 1992	Accession Number DSMZ 7101
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's (if) reference number	HX 1185-1 WO	International appl. No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 12 June 1992	Accession Number DSM 7102
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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Applicant's or agent's fi' reference number	HX 1185-1 WO	International app. ion No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 12 June 1992	Accession Number DSM 7103
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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Authorized officer Jesper Nielsen	Authorized officer

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Applicant's or agent's <sup>(1)</sup> reference number	HX 1185-1 WO	International app. No.	PCT/SE 94 / 00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 12 June 1992	Accession Number DSM 7104
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's reference number	HX 1185-1 WO	International application No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

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B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSMZ)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 26 February 1993	Accession Number DSMZ 7495
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's file reference number	HX 1185-1 WO	International application No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 26 February 1993	Accession Number DSM 7496
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
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Applicant's or agent's reference number	HX 1185-1 WO	International application No.	PCT/SE 94/00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSMZ)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 26 February 1993	Accession Number DSMZ 7497
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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Applicant's or agent's file reference number	IX 1185-1 WO	International app. ionNo.	PCT/SE 94 / 00160
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 03 March 1993	Accession Number DSM 7501
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

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Applicant's or agent's (if reference number) <b>IX 1185-1 WO</b>	International app. ion No. <b>PCT/SE 94 / 00160</b>
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## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>38</u> , line <u>5 - 15</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Deutsche Sammlung von Mikroorganismen (DSM)	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b D-3300 Braunschweig Federal Republic of Germany	
Date of deposit 03 March 1993	Accession Number DSM 7502
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g. EPC Rule 28(4), U.K. Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.	
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## CLAIMS

1. A nucleic acid molecule encoding a polypeptide which is a BSSL variant shorter than 722 amino acids, said BSSL variant  
5 comprising part of the amino acid sequence shown as residues 536-722 in SEQ ID NO: 3.
2. A nucleic acid molecule according to claim 1, wherein the said BSSL variant has a phenylalanine residue in its C-terminal  
10 position.
3. A nucleic acid molecule according to claim 1 or 2, wherein the said BSSL variant comprises the sequence Gln-Met-Pro in its C-terminal part.  
15
4. A nucleic acid molecule according to any one of claims 1-3, wherein the said BSSL variant comprises the amino acid sequence shown as residues 712-722 in SEQ ID NO: 3 in its C-terminal part.
- 20 5. A nucleic acid molecule according to any one of claims 1-4, wherein the said BSSL variant comprises less than 16 repeat units.
6. A nucleic acid molecule according to claim 1 which encodes a polypeptide, the amino acid sequence of which is at least 90%  
25 homologous with the amino acid sequence shown as SEQ ID NO: 5, 6 or 9 in the Sequence Listing.
7. A nucleic acid molecule according to claim 6 encoding a polypeptide comprising the amino acid sequence shown as SEQ  
30 ID NO: 5, 6 or 9 in the Sequence Listing.

8. A nucleic acid molecule which encodes a polypeptide, the amino acid sequence of which is at least 90% homologous with the amino acid sequence shown as SEQ ID NO: 7 in the Sequence Listing, with the exception for those nucleic acid molecules which encode polypeptides which have an asparagine residue at position 187.  
5
9. A nucleic acid molecule according to claim 8 encoding a polypeptide comprising the amino acid sequence shown as SEQ ID NO: 7 in the Sequence Listing.  
10
10. A polypeptide shown as SEQ ID NO: 5, 6, 7 or 9 in the Sequence Listing.
11. A polypeptide encoded by a nucleic acid sequence according to any one of claims 1-9.  
15
12. A polypeptide according to claim 10 or 11 in substantially pure form.  
20
13. A hybrid gene comprising a nucleic acid molecule according to any one of claims 1-9.
14. A replicable expression vector comprising a hybrid gene according to claim 13.  
25
15. A vector according to claim 14, which vector is the bovine papilloma virus vector pS258, pS259 or pS299.
16. A cell harbouring a hybrid gene according to claim 13.  
30

17. A cell according to claim 16, which cell is from the murine cell line C127 or from *E.coli*.
- 5 18. A process for the production of a recombinant polypeptide, said process comprising (i) inserting a nucleic acid molecule according to any one of claims 1-9 in a hybrid gene which is able to replicate in a specific host cell or organism; (ii) introducing the resulting recombinant hybrid gene into a host cell or organism; (iii) identifying and growing the resulting cell in or on a culture medium, or identifying and reproducing an organism, for expression of the polypeptide; and (iv) recovering the polypeptide.
- 10 19. A process according to claim 18 in which the hybrid gene is comprised in the bovine papilloma virus vector pS258, pS259 or pS299.
- 15 20. An expression system, comprising a hybrid gene which is expressible in a host cell or organism harbouring said hybrid gene, so that a recombinant polypeptide is produced when the hybrid gene is expressed, said hybrid gene being produced by inserting a nucleic acid sequence according to any of claims 1-9 into a gene capable of mediating expression of the said hybrid gene.
- 20 25 21. A process of producing a transgenic non-human mammal capable of expressing a BSSL variant, comprising (a) introducing an expression system according to claim 20 into a fertilized egg or a cell of an embryo of a non-human mammal so as to incorporate the expression system into the germline of the mammal and (b) developing the resulting introduced fertilized egg or embryo into an adult female non-human mammal.
- 30

22. A process of producing a transgenic non-human mammal capable of expressing a BSSL variant and substantially incapable of expressing BSSL from the mammal itself, comprising (a) destroying the BSSL expressing capability of the mammal so that substantially no mammalian BSSL is expressed and inserting an expression system according to claim 20 into the germline of the mammal in such a manner that a BSSL variant is expressed in the mammal; and/or (b) replacing the mammalian BSSL gene or part thereof with an expression system according to claim 20.
23. A transgenic non-human mammal harbouring in its genome a DNA sequence according to any one of claims 1-9.
24. A transgenic non-human mammal according to claim 23 in which the DNA sequence is present in the germline of the mammal.
25. A transgenic non-human mammal according to claim 23 or 24 in which the DNA sequence is present in a milk protein gene of the mammal.
26. A transgenic non-human mammal according to any one of claims 23-25 which is selected from the group consisting of mice, rats, rabbits, sheep, pigs and cattle.
27. Progeny of a transgenic non-human mammal according to any one of claims 23-26.
28. Milk obtained from a transgenic non-human mammal according to any one of claims 23-27.
29. An infant formula comprising milk according to claim 28.

30. An infant formula comprising a polypeptide according to any one of claims 10-12.
- 5 31. A process for production of an infant formula by supplementing an infant food formula with a polypeptide according to any one of claims 10-12.
32. Use of a polypeptide according to any one of claims 10-12 as a supplement to an infant food formulation.
- 10 33. A pharmaceutical composition comprising a polypeptide according to any one of claims 10-12.
- 15 34. A polypeptide according to any one of claims 10-12 for use in therapy.
35. Use of a polypeptide according to any one of claims 10-12 for the manufacture of a medicament for the treatment of a pathological condition related to exocrine pancreatic insufficiency.
- 20 36. The use according to claim 35 for the manufacture of a medicament for the treatment of cystic fibrosis.
- 25 37. The use according to claim 35 for the manufacture of a medicament for the treatment of chronic pancreatitis.
38. The use according to claim 35 for the manufacture of a medicament for the treatment of fat malabsorption.
- 30 39. The use according to claim 35 for the manufacture of a medicament for the treatment of malabsorption of fat soluble vitamins.

40. The use according to claim 35 for the manufacture of a medicament for the treatment of fat malabsorption due to physiological reasons.
- 5      41. The use according to claim 35 for the manufacture of a medicament for the improvement of the utilization of dietary lipids.
- 10      42. The use according to claim 35 for the manufacture of a medicament for the improvement of the utilization of dietary lipids in preterm born infants.

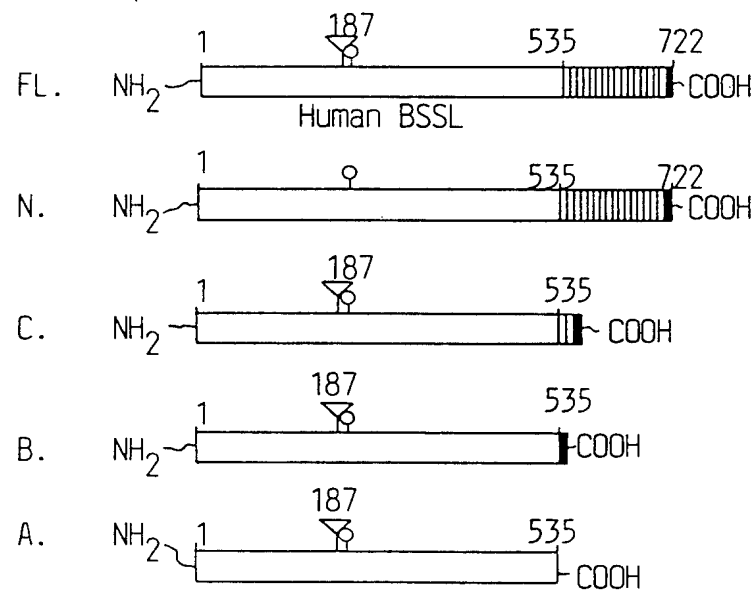
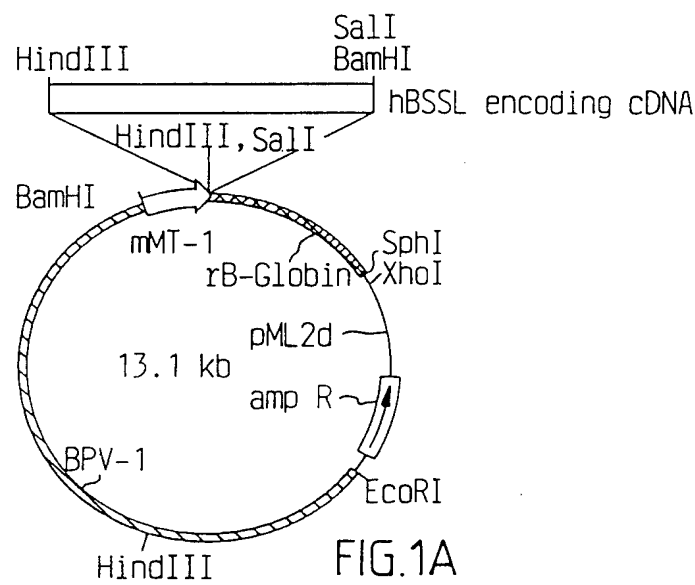


FIG.1B



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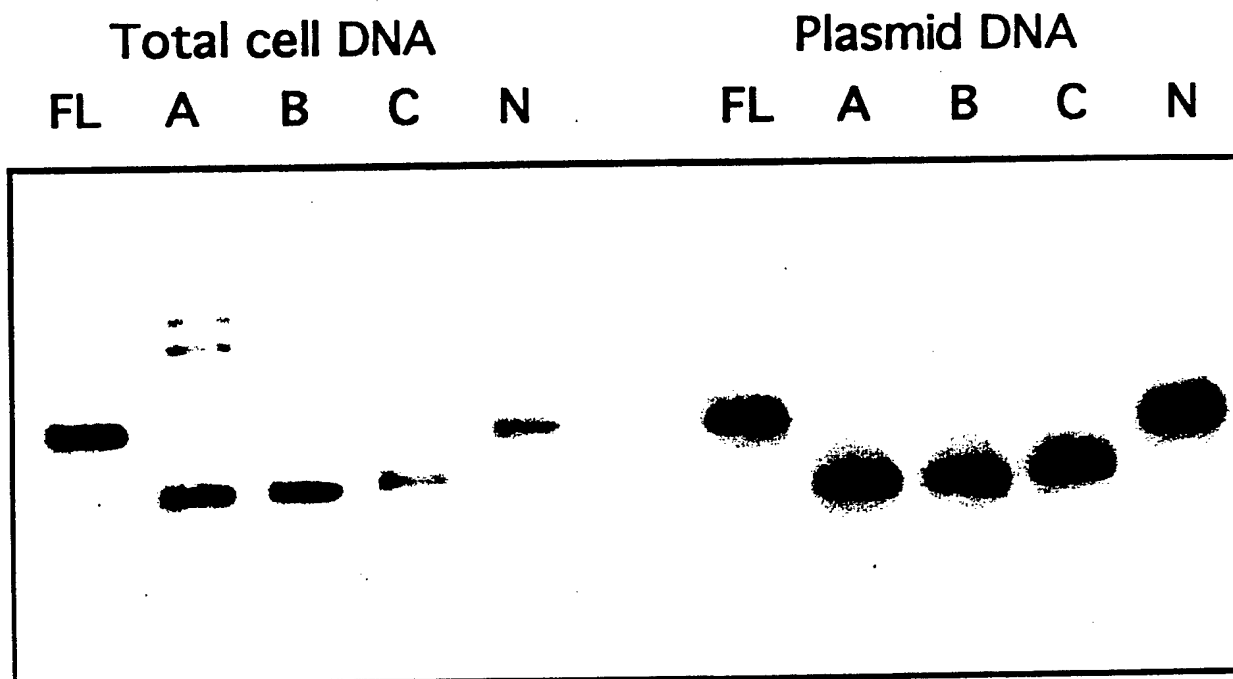


FIG. 2

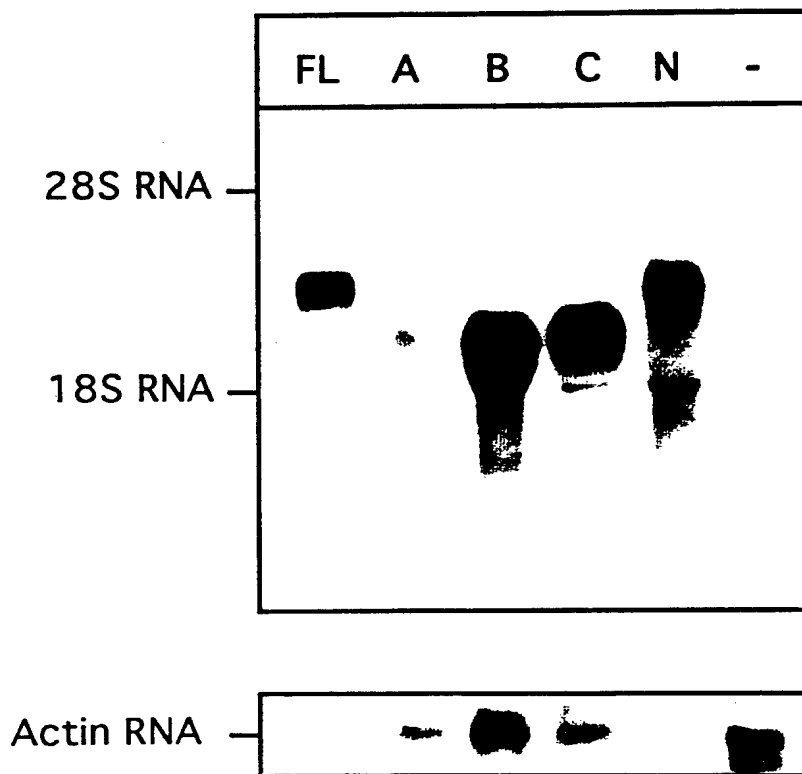
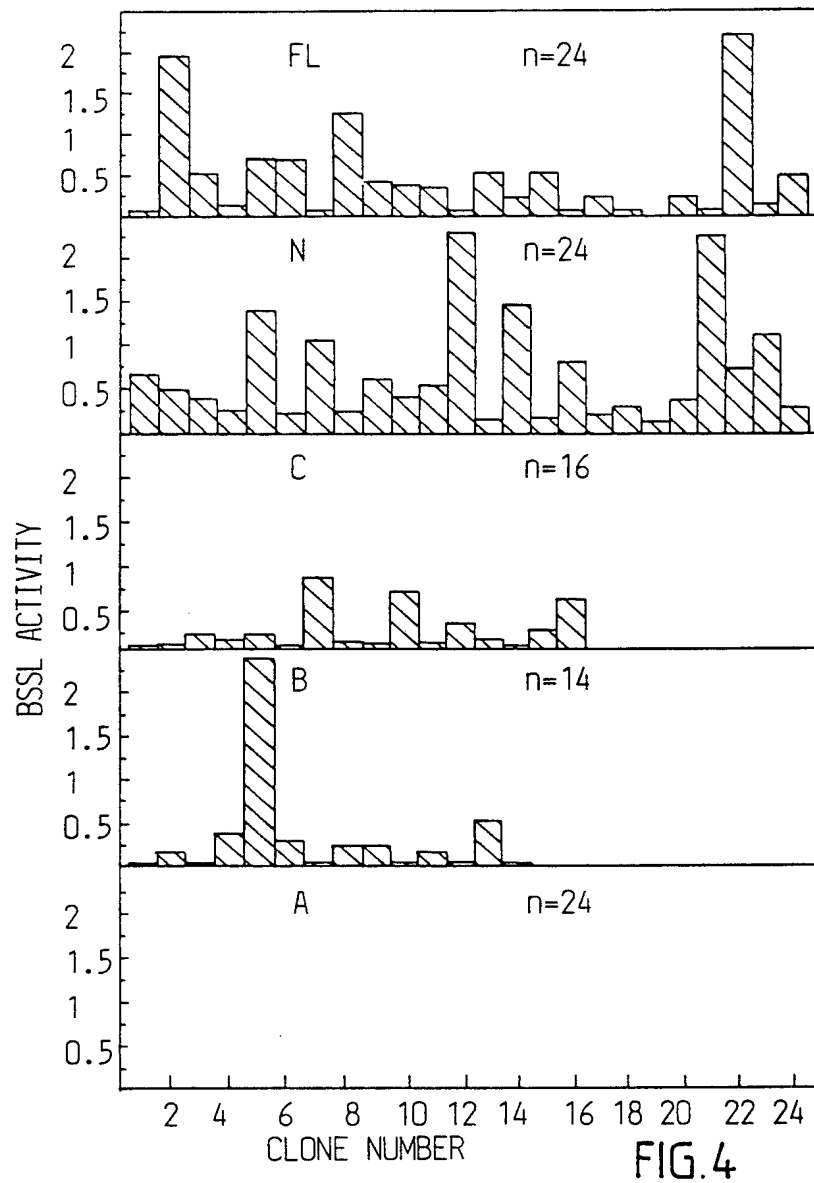


FIG. 3

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SUBSTITUTE SHEET

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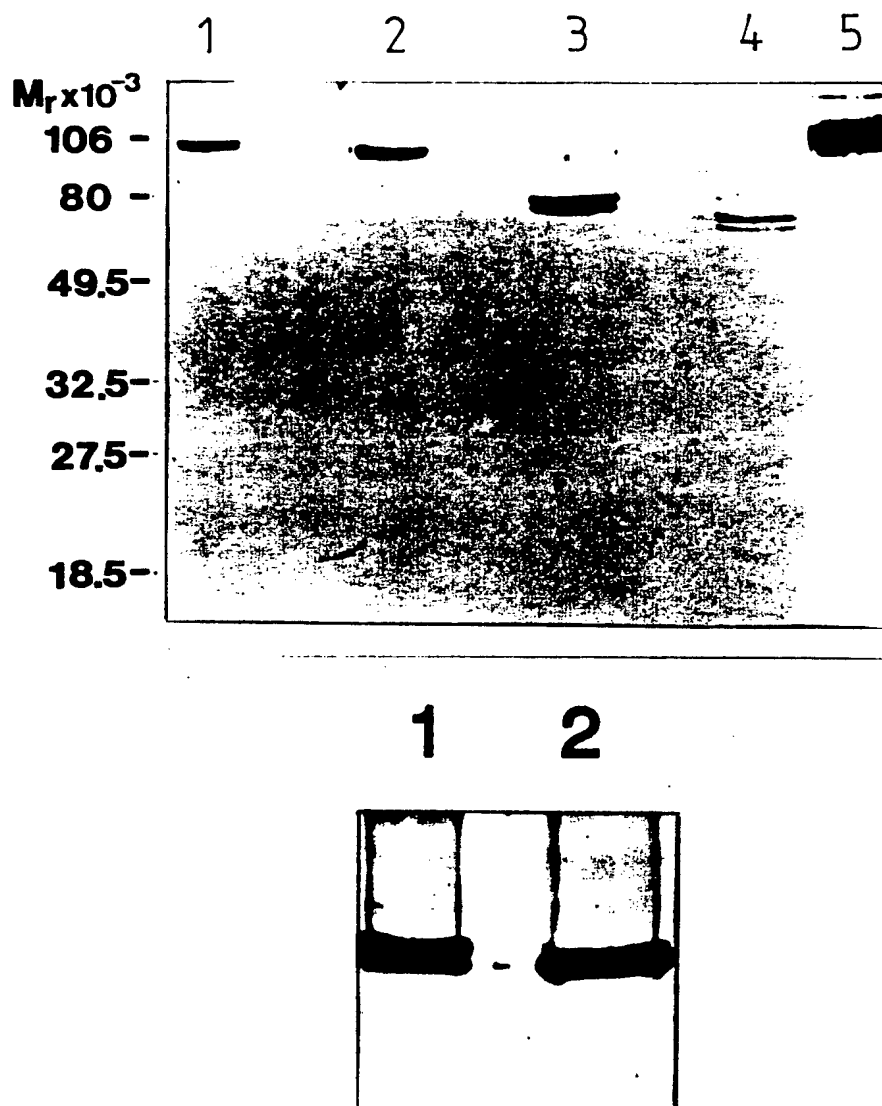


FIG. 5

SUBSTITUTE SHEET

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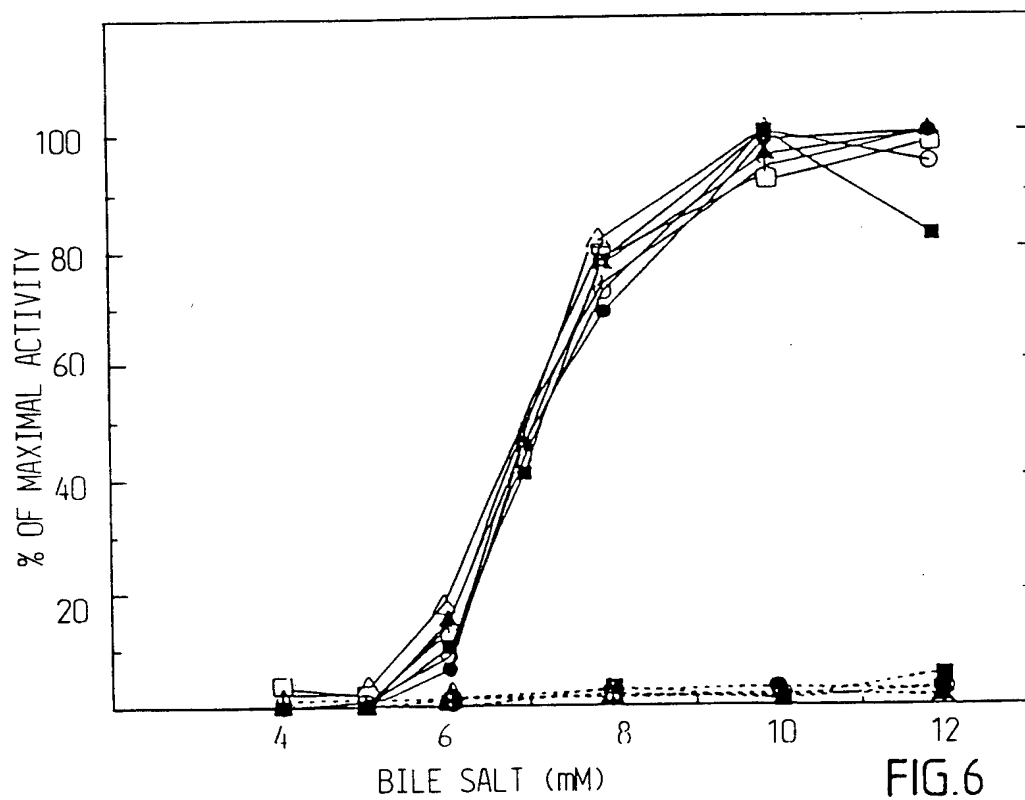


FIG. 6

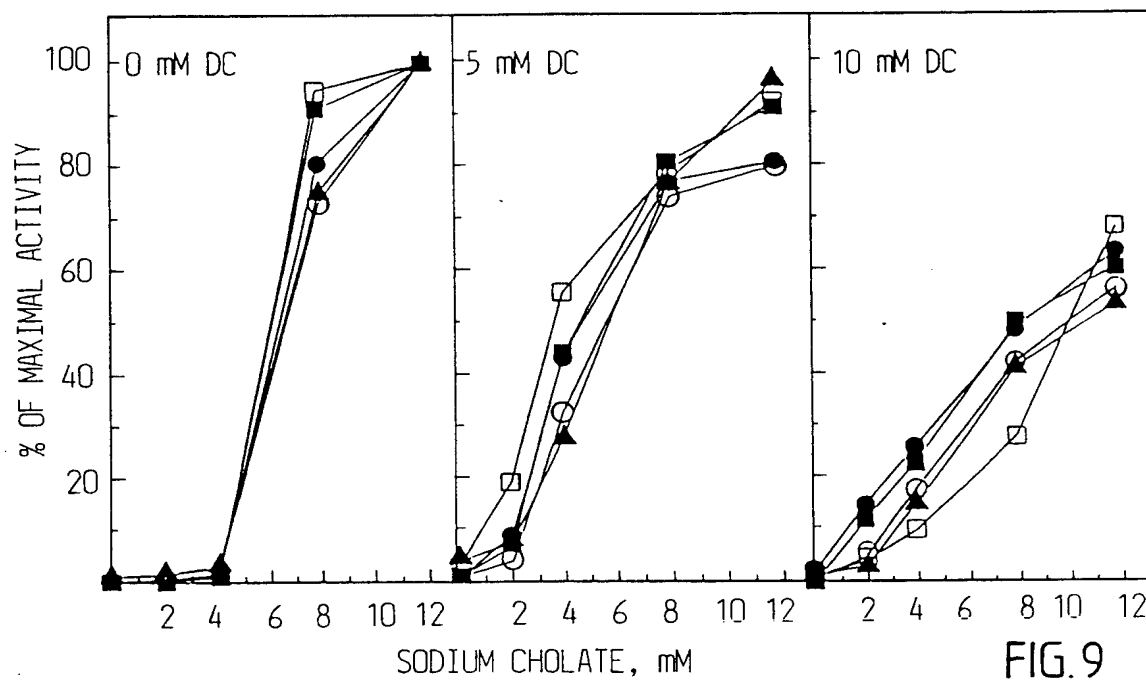
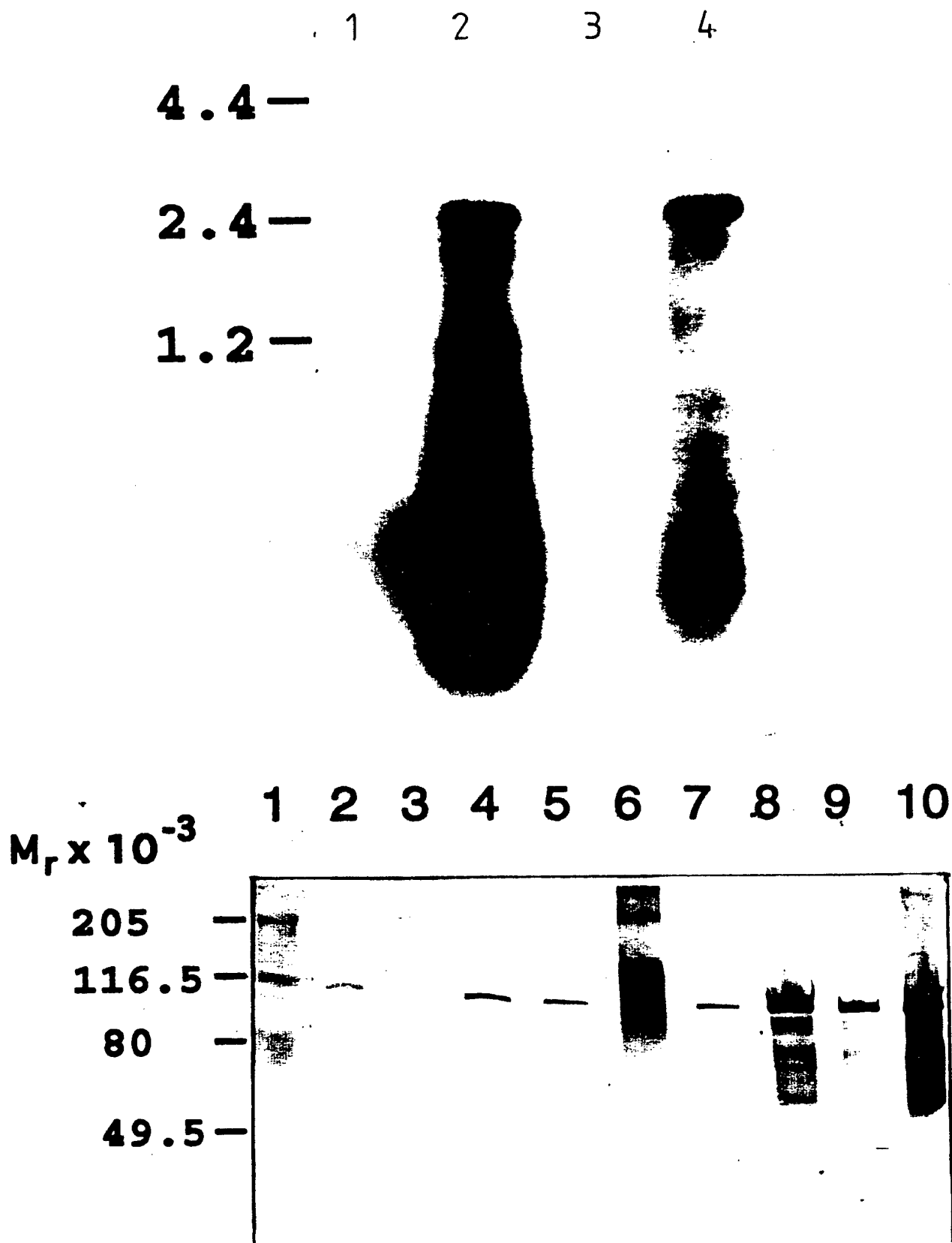


FIG. 9

SUBSTITUTE SHEET

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SUBSTITUTE SHEET

FIG. 7

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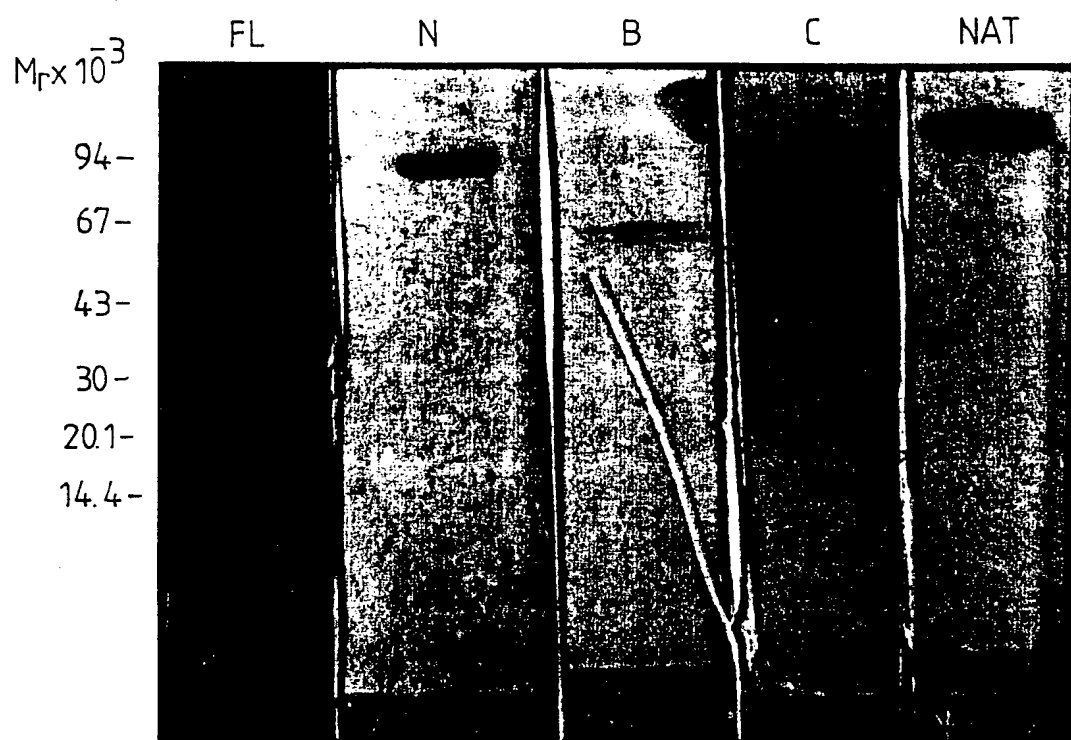
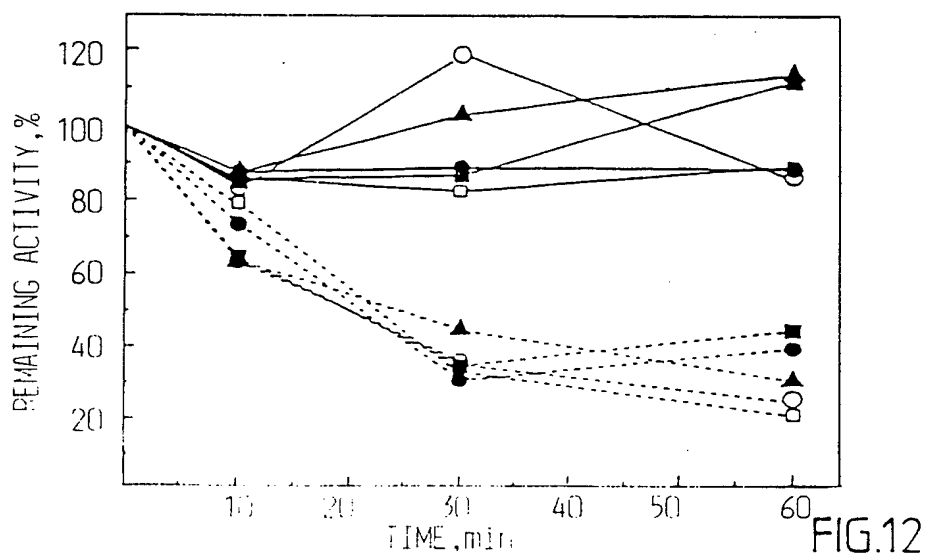
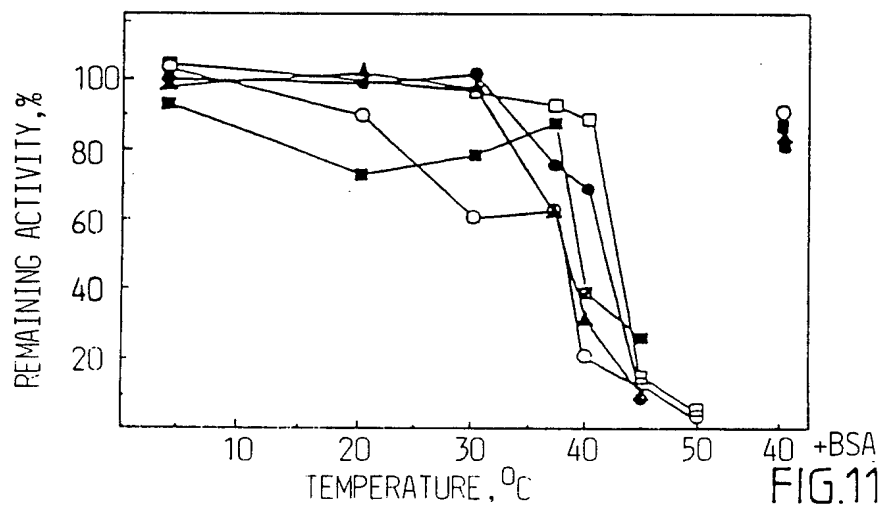
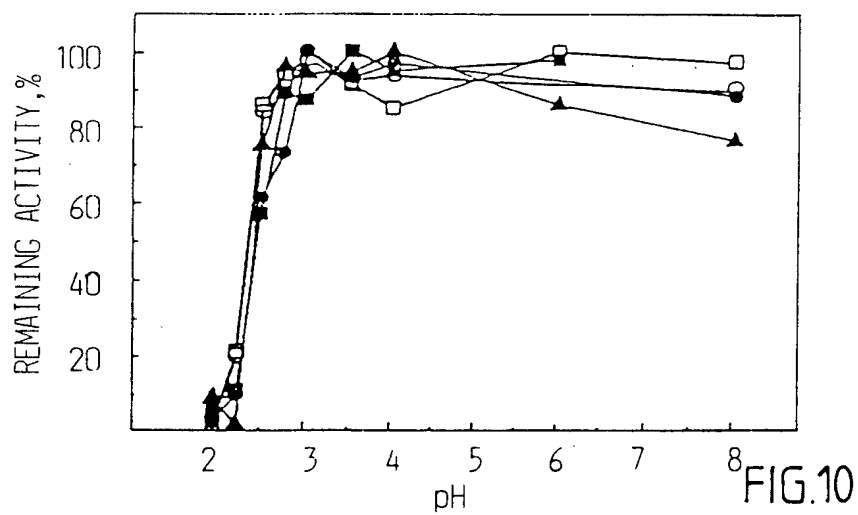


FIG.8

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SUBSTITUTE SHEET

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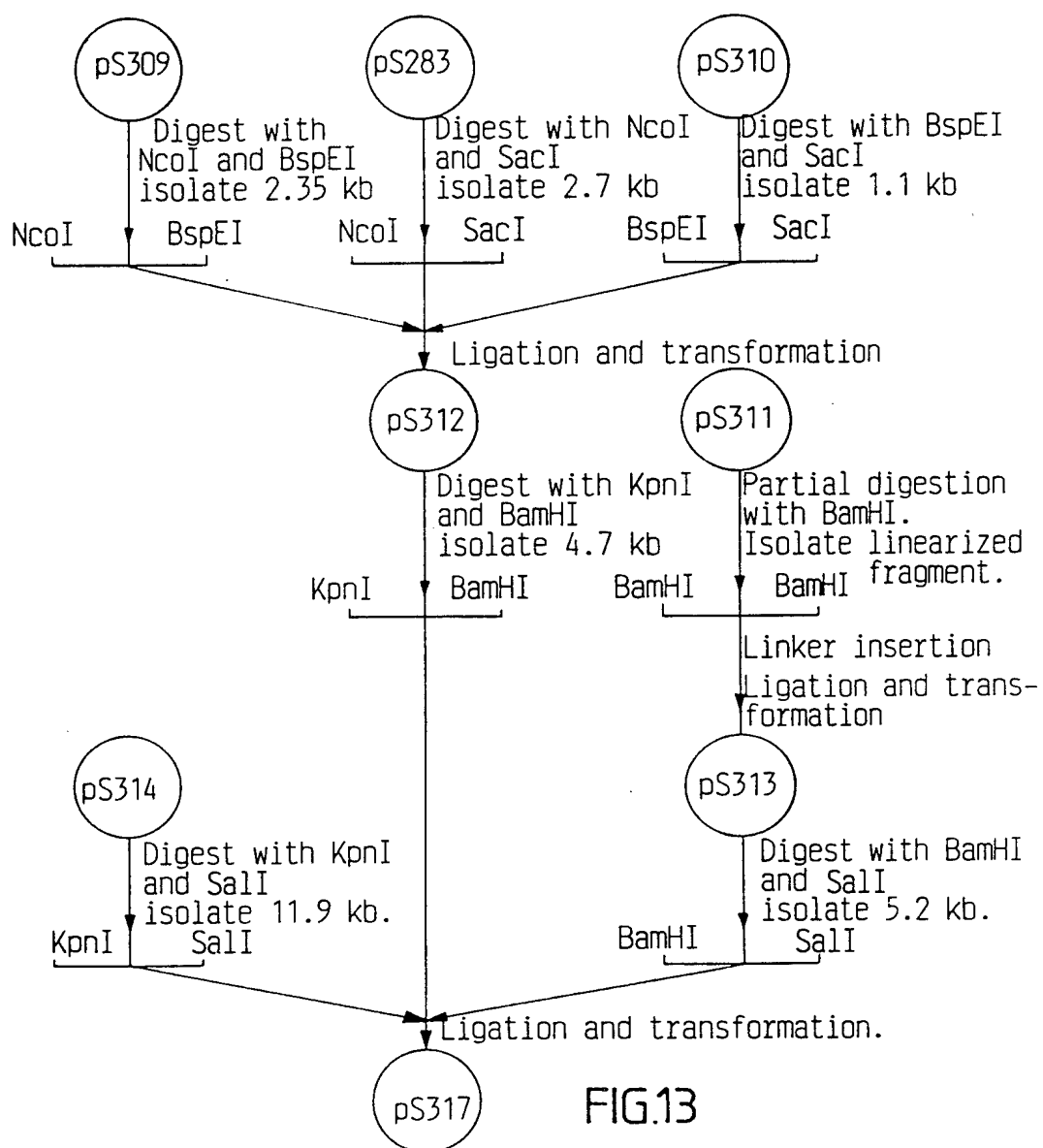
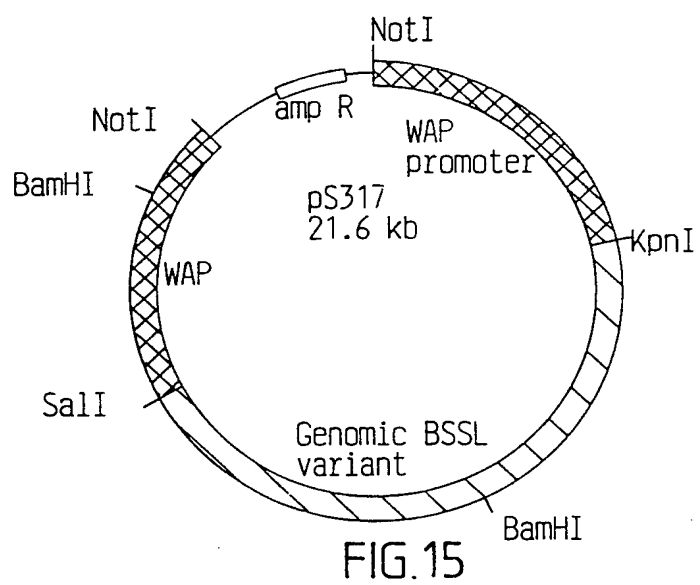
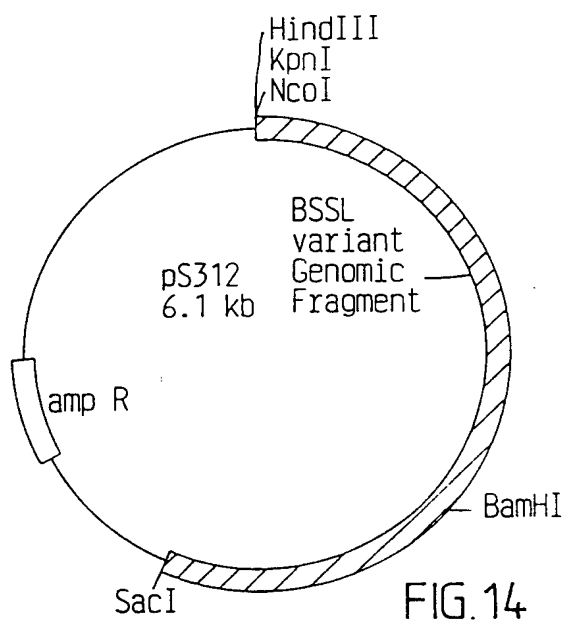


FIG.13



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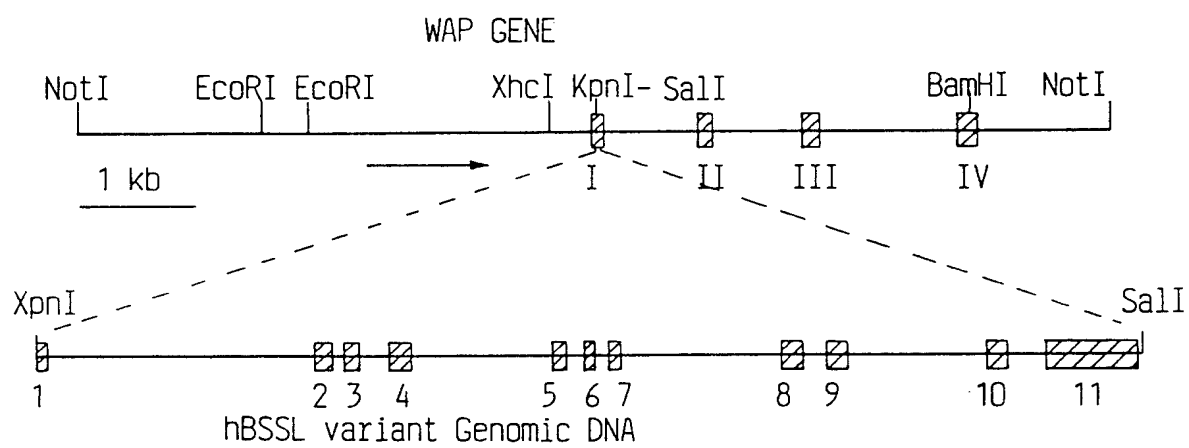


FIG.16

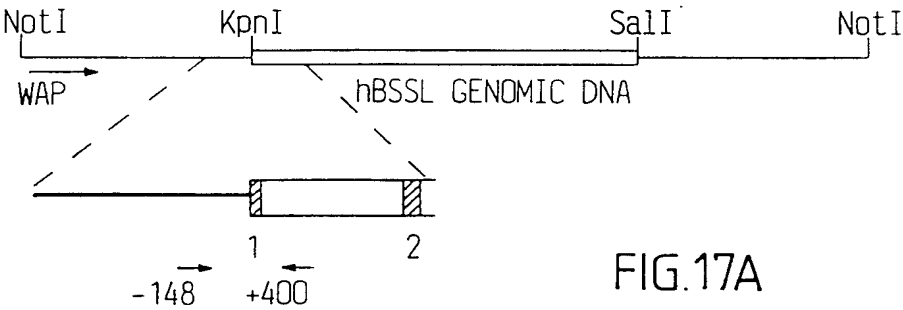


FIG.17A

Primer	Sequence ( 5' -3' )
5'-primer	CTGTGTGGCAAGAAGGAAGTGT
3'-primer	CAACTCCTGACCTCAAGTGATC

FIG.17B

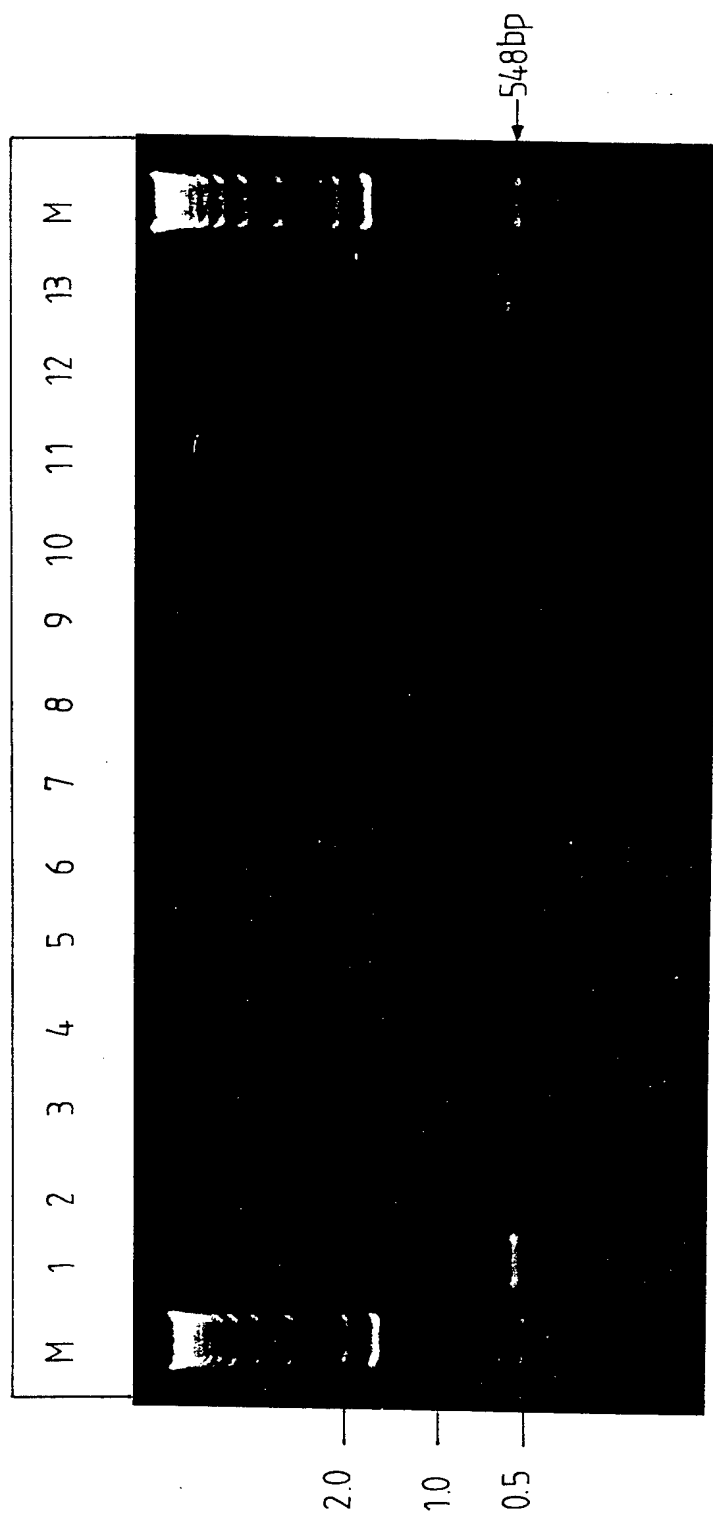
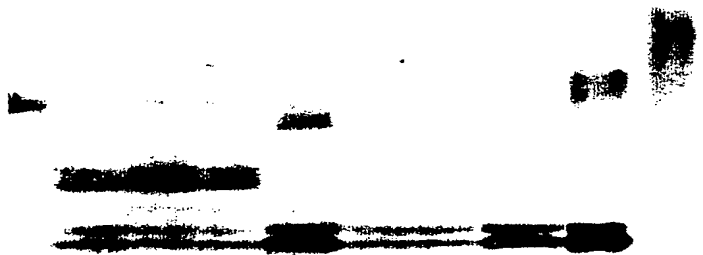
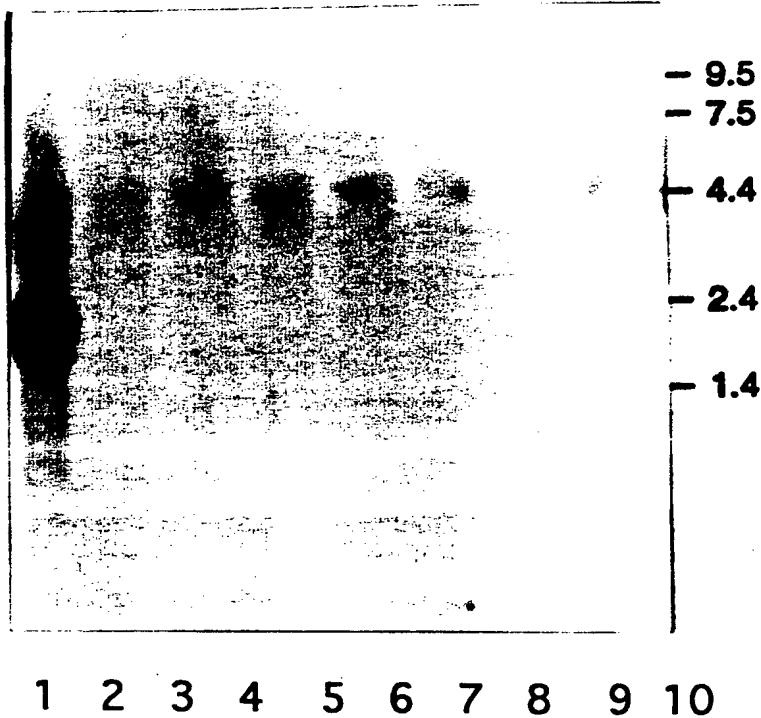


FIG.17C

**Mg Li Ki Sp He Lu Sg Br**



1  
INTERNATIONAL SEARCH REPORT

International application No.  
PCT/SE 94/00160

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC : C12N 9/20, A01K 67/027, C12N 15/00  
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC : C12N, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, WPI, CLAIMS, EMBL, PIRONLY, SWISSPROT, GENESEQ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A1, 9118923 (AKTIEBOLAGET ASTRA), 12 December 1991 (12.12.91)  --	1-42
A	WO, A1, 9115234 (OKLAHOMA MEDICAL RESEARCH FOUNDATION), 17 October 1991 (17.10.91)  --	1-42
A	Dialog Information Services, file 155: Medline, Dialog accession no.07487144, Medline accession no. 91006144, Nilsson J et al: "cDNA cloning of human-milk bile-salt-stimulated lipase and evidence for its identity to pancreatic carboxylic ester hydrolase", Eur J Biochem Sep 11 1990, 192 (2) p543-50  -----	1-42

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document but published on or after the international filing date	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search  9 June 1994	Date of mailing of the international search report  15 -06- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer  Jonny Brun Telephone No. +46 8 782 25 00

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

07/05/94

International application No.

PCT/SE 94/00160

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9118923	12/12/91	AU-A- 7964591 CN-A- 1064313 EP-A- 0535048	31/12/91 09/09/92 07/04/93
WO-A1- 9115234	17/10/91	EP-A- 0525076 US-A- 5200183	03/02/93 06/04/93